SYNTHESIS AND BIOLOGICAL ACTIVITY OF FARNESYL PYROPHOSPHATE ANALOGS

by

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Abstract. In this thesis are described the synthesis and biological activity of the pyrophosphates of the following farnesol analogs (numbers apply to the pyrophosphates): 2-methylfarnesol (15); 7,11-dimethyl-3-ethyl-2,6,10-dodecatrien-1-ol (17); 3-desmethylfarnesol (16); 4-thiomethylfarnesol (20); 7,11-dimethyl-3-iodo-2,6,10-dodecatrien-1-ol (18); 7,11-dimethyldodeca-2-iodo-2,6,10-dodecatrien-1-ol (19); 7,11-dimethyldodeca-6,10-dien-2-yn-1-ol (24); phytol (23); 3,7,11-trimethyl-2-dodecen-1-ol (22); 3,7,11-trimethyldodecan-1-ol (25); and geraniol (21). All the pyrophosphate analogs were found to inhibit the incorporation of tritium labeled farnesyl pyrophosphate into squalene by a yeast enzyme preparation, and are arranged above in decreasing order of inhibitory capacity. Kinetic analysis of the inhibition indicated that each analog is a competitive or mixed inhibitor. However, irreversible inhibition was not detected. Free alcohol precursors and 22 monophosphate did not inhibit the formation of squalene. The result of these studies indicate that attachment to the active site is determined mainly by pyrophosphate bonding forces, assisted by relatively nonspecific lipophilic interactions. Analogs which retain the C_{15} triply-unsaturated chain are in general more potent inhibitors than saturated analogs. Substitution at 2,3, and 4 positions, and increasing the chain length are well tolerated. Decreasing the chain length considerably reduces binding.

Tritium labeled analogs 15,16,17,20, and 22 were tested as pseudo substrates. Only 15 and 16 were accepted by the enzyme. These analogs were found to be incorporated as cosubstrates during the second catalytic step by tritium release experiments. The products obtained in these condensations were identified as 11-methylsqualene and 10-desmethylsqualene, respectively. The results of these studies indicate that the enzyme does not recognize a substrate without the C-3 methyl group during the first catalytic step. The effects of steric and electronic alterations around the 2,3, and 4 positions, as well as hydrogenation of the double bonds are disscused.

The synthesis and preliminary bioassay of prenyl substituted cyclobutanones as inhibitors of squalene synthetase were investigated. The cyclobutanones could not be prepared by photochemical reactions. The synthesis of these compounds was achieved by condensation of keteneimmonium cations with a prenyl diene. These cyclobutanones, and the alcohol pyrophosphate derived from one of them, were found to be weak inhibitors of the enzyme.

To my wife Rosario
and my daughter Rosario Elaine

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to Prof. P. R. Ortiz de Montellano for his invaluable guidance in the realization of this project and the development of my scientific awareness. I am grateful to Dr. Jeng Shu Wei and Dr. Si Myung Byun for teaching me the bioassay techniques and for their collaboration in this work. I express my sincere appreciation to Wayne A. Vinson, Amrit S. Boparai, and Dr. Charles K. Hsu for their collaboration in this project and the many hours of disscusions which have been of influence in my scientific development. I am thankful to the Facultad de Quimica de la Universidad Nacional Autonoma de Mexico and the Banco de Mexico for financial support. My heartfelt thanks are also due to my parents for the encouragement to continue my education. I am indebted to my profesors, friends, and to the people of San Francisco for their understanding and kindness. They have all extended to me a warm feeling of friendship and given me an enlighting experience of human values.

TABLE OF CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

1. Objectives

The biological synthesis of sterols from acetate has been largely elucidated during the last two decades.[1] An outline of this sequence as it is now known is given for the biosynthesis of squalene in Fig. 1. All the steps leading from acetate to farnesyl pyrophosphate have been studied in substantial detail and the enzymes involved in these transformations have been obtained in soluble and partially purified form.[1] More recently, significant progress has been made in the study of the farnesyl pyrophosphate coupling reaction catalyzed by squalene synthetase. However, the mechanism and active site specificity of this enzyme still remain open to investigation. Such studies are the concern of this thesis. It is hoped that this research will provide guidelines for the design of specific inhibitors of squalene synthetase, possibly of use as therapeutic agents in the treatment of cardiovascular diseases, which are closely correlated with high plasma cholesterol levels.[2]

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(1) For general reviews, see (a) I. D. Frantz and G. J. Schroepfer, Ann. Rev. Biochem., 36, 691 (1967); (b) R. B. Clayton, Quart. Rev., 19, 168 (1965); (c) J. W. Cornforth, ibid., 23, 125 (1969).

(2) National Heart and Lung Institute Task Force on Arteriosclerosis, "Arteriosclerosis", Department of Health, Education, and Welfare, Publ. No. 72-219, 1971. Vol. 2

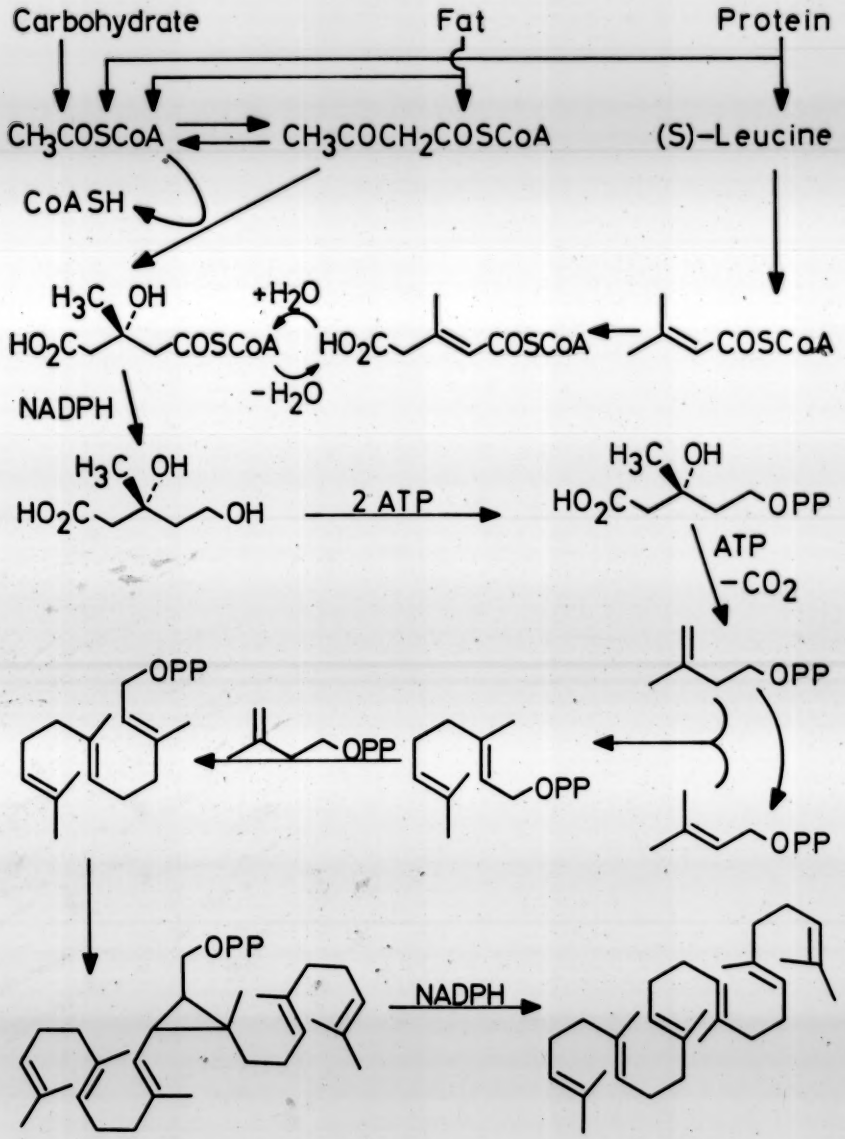
Carbohydrate
Fat
Protein
CH3COSCoA
CH3COCH2COSCoA
(S)-Leucine
CoA SH
H3C OH
HO2C
COSCoA
+H2O
-H2O
NADPH
2 ATP
OPP
ATP
-CO2

Fig.1

2. General Background

a. Overall Conversion of Farnesyl Pyrophosphate to Squalene

The net molecular changes involved in the biological conversion of farnesyl pyrophosphate 1 to squalene 2 have been the object of a series of brilliant investigations by Popják, Cornforth, and their co-workers. They demonstrated that this transformation is, as shown in Fig. 2, an asymmetric process.[1] Although a head-to-head coupling occurs, one of the four hydrogen atoms on the two central carbons of squalene does not derive from the precursor farnesyl but comes instead from the β-face of the pyridine ring in NADPH.[3,4] This hydrogen exchange involves the stereospecific replacement of a pro-s hydrogen on C-1 of farnesol by the nucleotide hydride in such a way that no change of configuration is observed in the corresponding carbon of squalene.[3a,5] On the other hand the configuration is inverted at C-1 of the farnesyl unit not involved in hydrogen exchange during the condensation. These investigations were

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(3) (a) J. W. Cornforth, R. H. Cornforth, C. Donninger, G. Popják, G. Ryback, and G. J. Schroepfer, Proc. Royal Society (B), 163, 436 (1966); (b) G. Popják, D. S. Goodman, J. W. Cornforth, R. H. Cornforth, and R. Ryhage, J. Biol. Chem., 236, 1934 (1961); (c) G. Popják, J. W. Cornforth, R. H. Cornforth, R. Ryhage, and D. S. Goodman, ibid., 237, 56 (1962).

(4) C. R. Childs and K. Bloch, J. Biol. Chem., 237, 62 (1962).

(5) J. W. Cornforth, R. H. Cornforth, C. Donninger, G. Popják, G. Ryback and G. J. Schroepfer, Biochem. Biophys. Res. Commun., 11, 125 (1963).

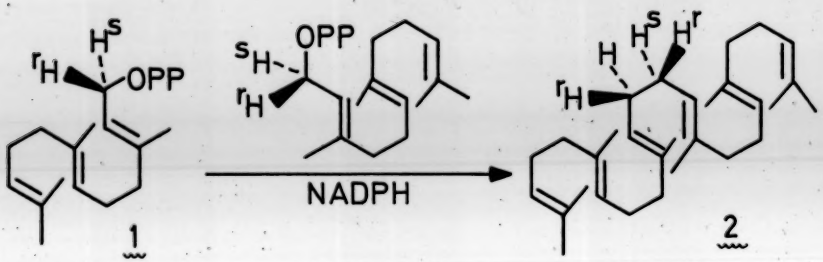

Fig.2

carried out with insoluble yeast and liver enzyme preparations.[6,7]

b. Presqualene pyrophosphate

Omission of NADPH from incubation mixtures led Rilling, in 1966, to the isolation of a thirty carbon pyrophosphate intermediate, now known as presqualene pyrophosphate.[8] The isolated mono-pyrophosphate was shown to retain only three of the four C-1 protons from its farnesol precursors and was readily converted to squalene on incubation with enzyme and NADPH.[8] After considerable controversy[9] the chemical structure of this intermediate was finally established

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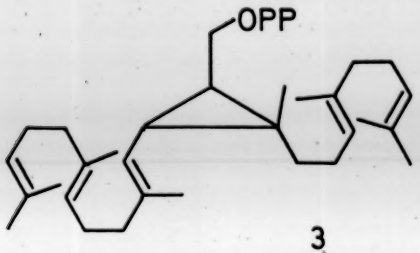
(6) See ref. 1 - 5

(7) D. S. Goodman and G. Popják, J. Lipid Res., 1, 286 (1960).

(8) H. C. Rilling, J. Biol. Chem., 241, 3233 (1966).

(9) (a) G. Popják, J. Edmond, K. Clifford, and V. P. Williams, J. Biol. Chem., 244, 1897 (1969); (b) H. C. Rilling and W.W. Epstein, J. Am. Chem. Soc., 91, 1041 (1969); (c) W. W. Epstein and H. C. Rilling, J. Biol. Chem., 245, 4597 (1970); (d) H. Wasner and F. Lynen, Fed. Eur. Biochem. Soc. Lett., 12, 54 (1970); (e) J. Edmond, G. Popják, S. M. Wong, and V. P. Williams, J. Biol. Chem., 246, 6254 (1971).



3

as 3 by physico-chemical methods and by chemical synthesis.[10] Recently, presqualene pyrophosphate has also been isolated from intact rat liver and from a yeast microsomal preparation in the presence of NADPH,[11] establishing that this sterol precursor is not an artifact of NADPH deprivation.

c. Studies on Purified Squalene Synthetase

Very recently the solubilization and partial purification of squalene synthetase from yeast have been reported.[12] This exciting new development has clarified many questions regarding the nature of the enzyme and its mode of catalysis.

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(10) (a) H. C. Rilling, C. D. Poulter, W. W. Epstein, and B. Larsen, J. Am. Chem. Soc., 93, 1783 (1971); (b) L. J. Altman, E. C. Kowersky, and H. C. Rilling, ibid., 93, 1782 (1971); (c) R. M. Coates and W. H. Robinson, ibid., 93, 1785 (1971).

(11) F. Muscio, J. P. Carlson, L. Kuehl, and H. C. Rilling, J. Biol. Chem., 249, 3746 (1974).

(12) A. A. Qureshi, E. D. Beytia, and J. W. Porter, Biochem. Biophys. Res. Commun., 48, 1123 (1972); (b) A. A. Qureshi, E. D. Beytia, and J. W. Porter, J. Biol. Chem., 248, 1848 (1973); (c) I. Shechter and K. Bloch, ibid., 246, 7690 (1971).

The purified enzyme has been resolved into two components of different molecular weight. One of these, a polymeric unit, catalyzes the overall conversion of farnesyl pyrophosphate to squalene; the other, a protomeric unit, only catalyzes the formation of presqualene pyrophosphate. Each of these components is converted in part to the other under appropriate conditions.[12a,b] The general properties of the yeast enzyme are similar to those of the enzyme obtained from mammalian liver. A divalent metal ion, such as Mg^{++} or Mn^{++}, is essential for the formation of presqualene pyrophosphate, but probably not for the conversion of this compound to squalene. However, the rate of the latter reaction is greatly increased in the presence of Mg^{++}.[13]

NADH can replace NADPH as electron donor, although the reaction proceeds at a much lower rate.[12c, 13] Yeast squalene synthetase activity is inhibited by concentrations above 50 μM of farnesyl pyrophosphate, by N-ethylmaleimide, and, to a lesser extent, by iodoacetamide.[13] Hence, it appears that sulfhydryl groups are involved in either the actual catalytic function or in maintaining the protein conformation necessary for that function.

Initial velocity studies for the formation of squa-

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(13) E. Beytia, A. A. Qureshi, and J. W. Porter, J. Biol. Chem., 248, 1856 (1973).

lene and presqualene pyrophosphate from farnesyl pyrophosphate have shown that the mechanism of the condensation reaction is "ping-pong" (Fig. 3); thus, the first molecule of farnesyl pyrophosphate (FPP) binds to the enzyme (E) to form a complex (E-FPP) that releases inorganic pyrophosphate (PP) before binding another molecule of farnesyl pyrophosphate. Binding of the second molecule of farnesyl pyrophosphate gives the enzyme-presqualene pyrophosphate complex (E-PSQPP), which regenarates the enzyme on releasing presqualene pyrophosphate.[13]

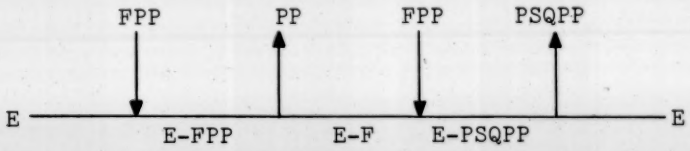

Fig. 3

For the second step of the reaction, the conversion of presqualene pyrophosphate to squalene, the binding sequence is different. Initial velocity kinetics and product inhibition studies have shown that this reaction is sequential ordered.[13] In the reduction reaction NADPH is the first substrate to bind to the enzyme, followed by presqualene pyrophosphate. The first product to be released is inorganic pyrophosphate, followed by squalene. The last product to leave the enzyme is NADP.[13] The postulated sequence is shown in Fig. 4.

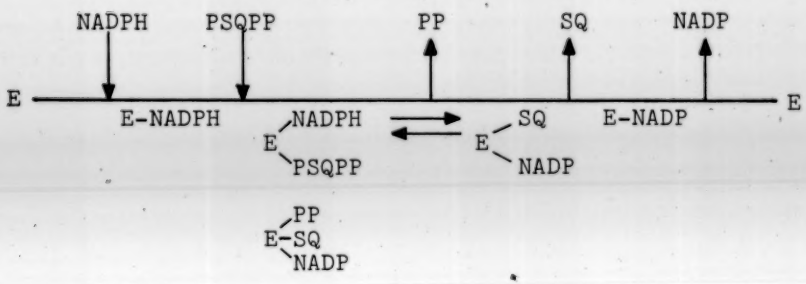

Fig. 4

The two kinetic mechanisms just discussed are derived from studies on the isolated half reactions. The connection between these two kinetic sequences in the overall biosynthetic transformation, however, is as yet not known. It is possible that presqualene pyrophosphate is not usually released during the overall enzymatic conversion of farnesyl pyrophosphate to squalene.

d. Mechanistic Speculation on Squalene Biosynthesis.

Studies on the biosynthesis of squalene have been simplified by division into two steps. These steps are the synthesis of presqualene pyrophosphate (3) from farnesyl pyrophosphate (1), with loss of one pyrophosphate moiety and one proton, and subsequent transformation of 3 to squalene (2), with incorporation of a hydride ion and loss of the second pyrophosphate group (see Fig. 5).

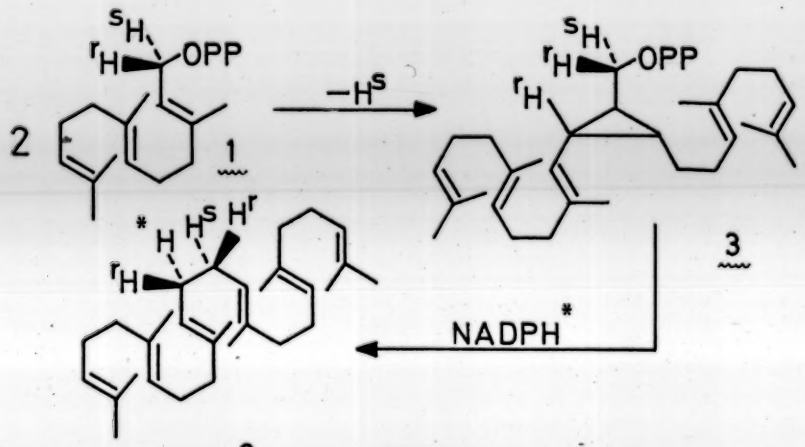

Fig.5

Taking into consideration all the observations discussed before, any mechanism postulated for the first step must account for nucleophilic displacement of a pyrophosphate group, nucleophilic or electrophilic addition to a double bond, proton removal, and cyclopropane formation.

Although numerous possibilities can be written, only a few of the most reasonable variants will be discussed here.

Several mechanisms have been proposed for the formation of presqualene pyrophosphate[9c-e, 10a] which, however, imply a sequential addition of farnesyl pyrophosphate units. A hypothetical mechanism that accounts for all the observations discussed, including the "ping-pong" reaction sequence,[13] is shown in Fig. 6. Elimination of the pyrophosphate group could proceed with rearrangement of the double bond to give 5 (Sn2'), or it could be a direct Sn2 displacement by a nucleophilic group on the enzyme, yielding farnesyl-

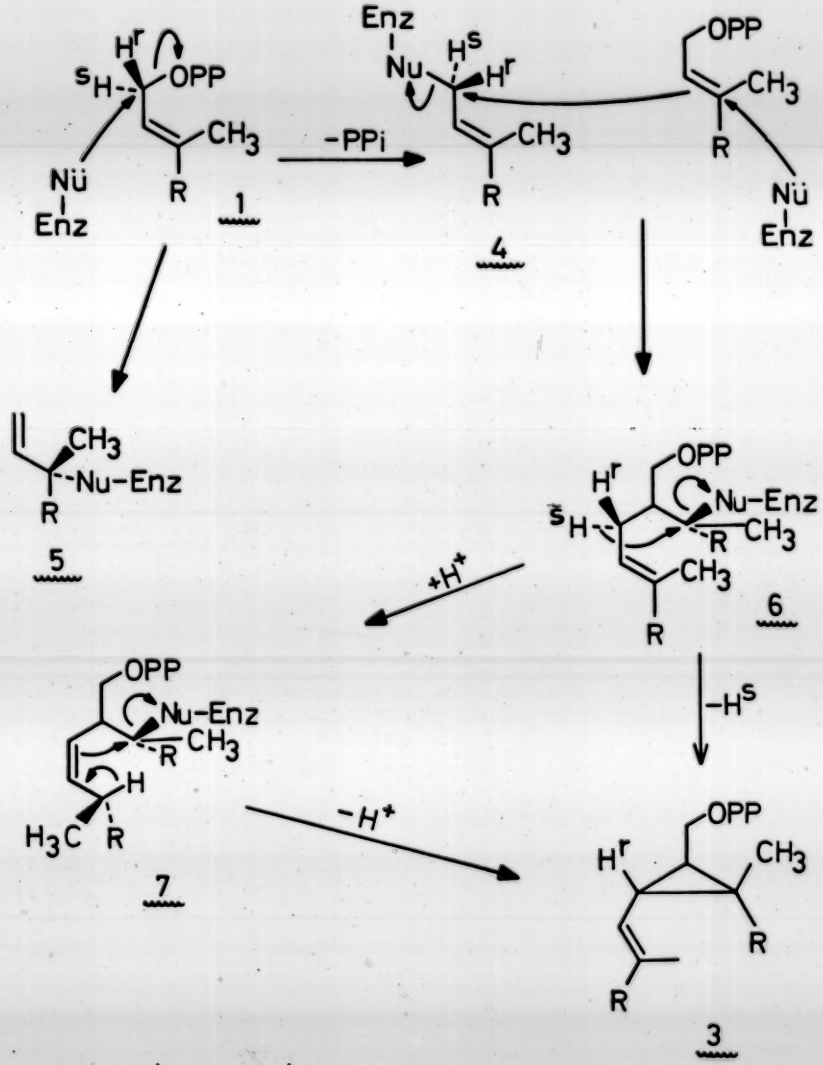

Fig.6

squalene synthetase complex 4. The π system at C-2 of a second molecule of farnesyl pyrophosphate could then displace the enzyme from 4 or 5, giving 6. This displacement could be assisted by a second nucleophilic group on the enzyme. The net effect of this double displacement is retention of the configuration around C-1 of both of the original molecules of farnesyl pyrophosphate. The nucleophilic groups on the enzyme might be sulfhydryl moieties. This would be consistent with the inhibitory action of -SH reagents such as N-ethylmaleimide. Stereospecific removal of H^S from 6 could lead to presqualene pyrophosphate by direct intramolecular displacement of the enzyme or, indirectly, through 7, as postulated by Van Tamelen and Schwartz.[14]

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(14) E. E. Van Tamelen and M. A. Schwartz, J. Am. Chem. Soc., 93, 1780 (1971).

An interesting mechanism of the type represented by structure 5 is outlined in Fig. 7.

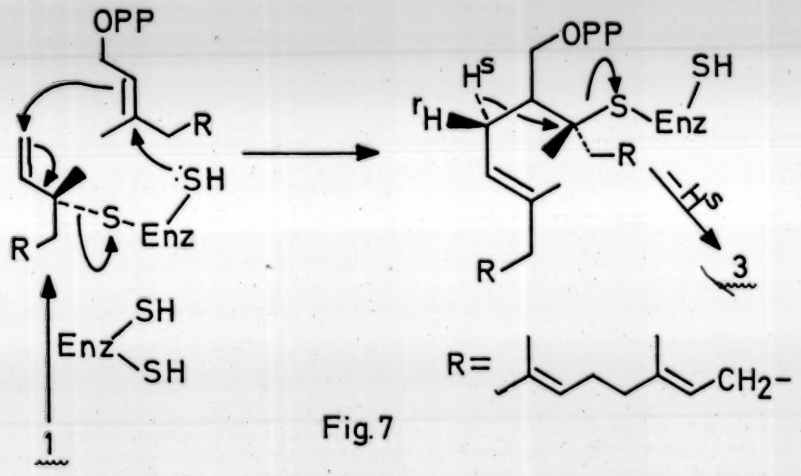

Fig.7

Of interest in this mechanism is the strong orienting influence which could be exerted by the enzyme. Thus, the sequence shown in Fig. 7 requires strong participation by the enzyme at both reacting centers, and thereby helps to rationalize the enzyme catalytic action.

An alternative possibility for direct displacement by an enzyme moiety would utilize the sulfonium salt 8 (Fig. 8). Generation of the sulfur stabilized ylide and addition to the double bond[15] would produce 9. It is highly probable that such a mechanism would require activation of the double bond by a group y^+, since sulfur ylid additions across double bonds to give cyclopropanes nor-

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(15) (a) H. Konig, Fortschritte der Chem. Forschung, 9, 487 (1968); (b) J. C. Bloch, Ann. Chim., 419 (1965).

mally occur with activated olefins.[15] A mechanism of this type would comply with all the stereochemical requirements.

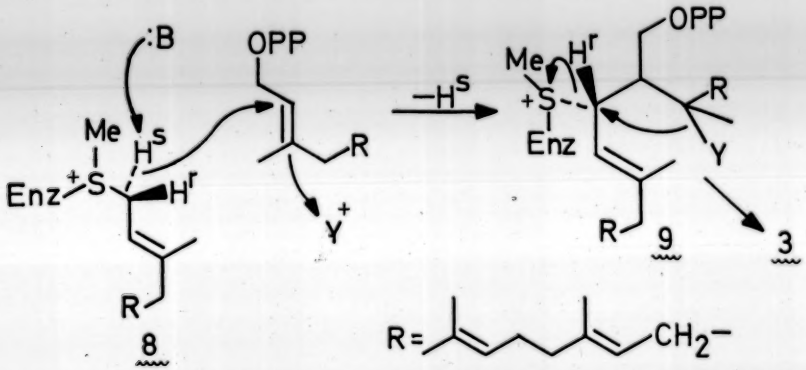

Fig.8

Several mechanisms have been proposed for the conversion of presqualene pyrophosphate to squalene. All of them derive from solvolysis studies with small (ten carbon) model compounds.[16] A recent evaluation of these mechanisms is summarized in Fig. 9.[17] For purposes of clarity the cations are represented in terms of classical structures.

The event initiating the rearrangements is loss of the pyrophosphate moiety to give the cyclopropylcarbinyl cation 10.[10a-c,15] The greatest difficulty occurs at the

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(16) (a) 10a-c; (b) 15; (c) R. M. Coates and W. H. Robinson, J. Am. Chem. Soc., 94, 5920 (1972); (d) C. D. Poulter, O. J. Muscio, C. J. Spillner, and R.G. Goodfellow, ibid., 94, 5921 (1972).

(17) (a) C. D. Poulter, J. Agr. Food Chem., 22, 167 (1974); (b) C. D. Poulter, O. J. Muscio, and R. J. Goodfellow, Biochem., 13, 1530 (1974).

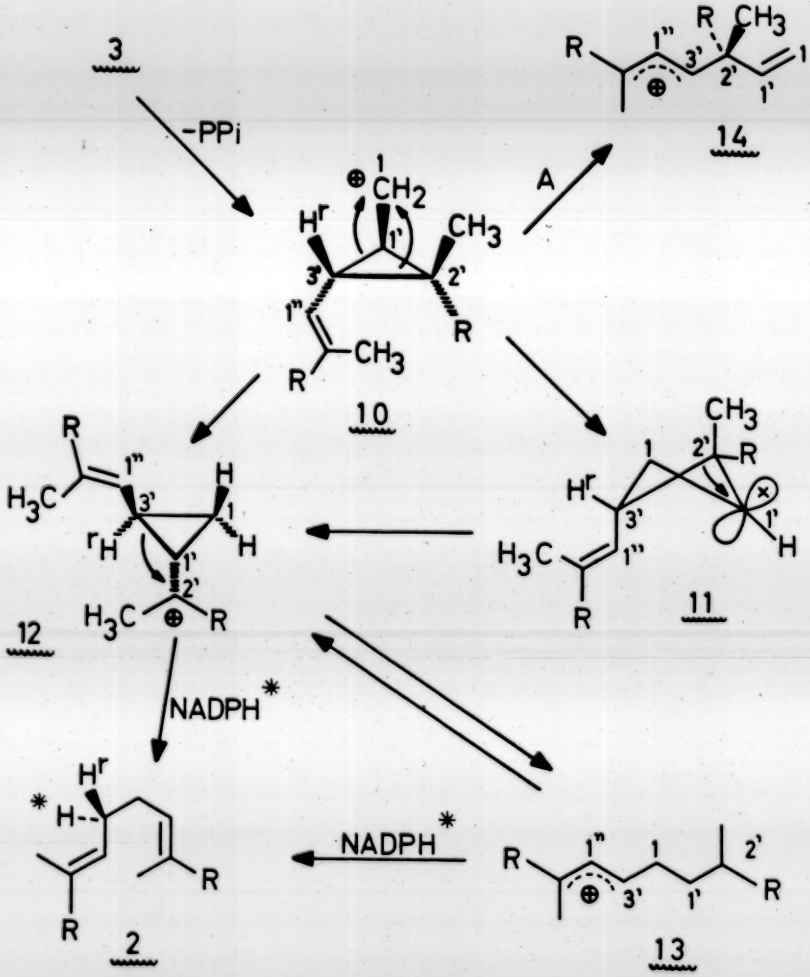

Fig.9

rearrangement of 10 to cyclobutyl cation 11, or directly to 12, since the thermodynamically favored pathway (A) leads to the formation of compounds derived from 14. These do not contain the squalene skeleton. It has been shown that once 11 is formed it quickly rearranges to tertiary cyclopropylmethylene cation 12 and that this entity is in partial equilibrium with allylic cation 13 which has the desired head-to-head carbon skeleton.[17] If the model mechanisms have validity for the enzymatic transformation the role of the enzyme would appear to be chiefly prevention of the thermodynamically favored rearrangement and catalysis of the final hydride transfer. There are two hypotheses concerning the former function. Coates and Robinson[16c] suggest that the plane of the adjacent double bond of 3 is fixed within the active site so that the π orbitals are aligned perpendicular to the C_1,-C_3, cyclopropane bond. Since allylic resonance stabilization of the incipient positive charge would not be possible in this conformation, premature ring opening would be avoided. Poulter et al.,[17] suggest that the substrate and coenzyme are bound before the reaction begins. Presqualene pyrophosphate is oriented in the active site such that the C_2,-C_3, cyclopropane bond is trans to the C_1-oxygen in order to account for the expected inversion of configuration at C_1. Heterolysis of the C_1-oxygen in the bound substrate then gives an intimate ion pair. The rearrangements to give squalene would then be directed by electrostatic interaction of the inti-

mate ion pairs, the pathway resulting in the smallest separation of charge being favored.

Although both suggestions offer a logical explanation, direct evidence on the enzymatic reaction still does not exist.

3. Nature and Order of Presentation

The experimental work is presented in the next chapter in three sections. Part A describes a study of structure-activity relationships which govern inhibition of squalene synthetase by farnesyl pyrophosphate analogs. Part B deals with a study of some of these anologs as enzyme substrates. In part C, an investigation of possible routes to the synthesis of a postulated transition state analog are described.

CHAPTER 2

Introduction

In order to define more clearly the properties of squalene synthetase and the requirements for binding of molecules to the active site (s), a study was made of the enzyme using farnesyl pyrophosphate analogs selected according to the criteria below:

1. Compounds that would help to define the minimun structural requirements for catalytic acceptance by the enzyme.

2. Compounds that would help to establish the maximum structural variations compatible with acceptance as substrates.

3. Compounds that could irreversibly inhibit the enzyme by blocking condensation of the second farnesyl pyrophosphate molecule after being themselves covalently bound.

4. Compounds with potential irreversible inhibitory capacity due to interference with the catalytic mechanism at the proton removal stage.

The analogs of farnesyl pyrophosphate studied are listed in Fig. 10. All the studies using analog 22, including its synthesis, were carried out in the same laboratory by A. S. Boparai.[18] These results are briefly discussed

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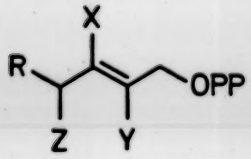
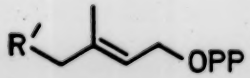
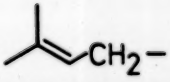
(18) P.R. Ortiz de Montellano, J. S. Wei, R. Castillo, C. K. Hsu, and A. Boparai, J. Med. Chem., in press.

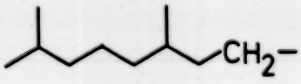




	X	Y	Z
1	Me	H	H
15	Me	Me	H
16	H	H	H
17	Et	H	H
18	I	H	H
19	H	I	H
20	Me	H	MeS

R′

21 

22 

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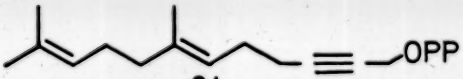

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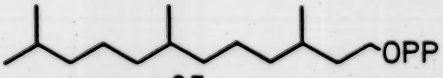

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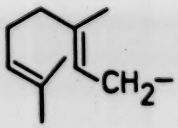
R = geranyl = 

OPP = pyrophosphate

Fig.10

here since they are pertinent to the general conclusions of this thesis. The precursor of 20, 4-thiomethylfarnesol, was synthesized by Dr. C. K. Hsu.[18]

All of the analogs meet at least one of the first three criteria. However, pyrophosphates 19, 20, and 24 are also of particular interest as possible irreversible inhibitors.

It has been noted[19] that replacement of a double bond with an acetylenic group, such as in 24, may be a general approach to the inactivation of enzymes that abstract protons from the carbon adjacent to the acetylenic linkage. The specificity of this inactivation process rests not merely on structural similarity to normal substrates but also upon the mechanism of the catalytic process. The acetylenic group is activated as a result of specific enzymatic action on the inhibitor molecule. Based upon earlier work, Morisaki and Bloch[20] suggested that this inactivation probably proceeds by rearrangement of the acetylenic function to an allene. It has also been noted[21] that, if the proposed mechanism is correct, other substrates which can form allenes during the catalytic process could also inactivate the enzyme.

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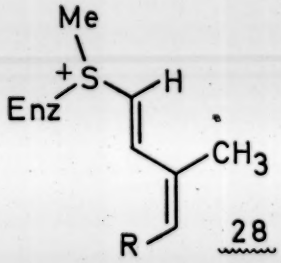
(19) R. H. Abeles and C. T. Walsh, J. Am. Chem. Soc., 95, 6124 (1973).

(20) M. Morisaki and K. Bloch, Biochem., 11, 309 (1972).

(21) R. C. Hevey, J. L. Maycock, and R. H. Abeles, J. Am. Chem. Soc., 95, 6125 (1973).

For analog 24, normal reaction might lead to formation of allene 26 or 27 (Fig. 11). In both cases removal of a proton from the C-1 carbon of the propargyl unit would give a sulfur stabilized anion, which could protonate to give an allene rather than undergoing cyclopropane ring closure. In the case of analog 20, the anion formed by proton removal could undergo iodide elimination to the same allene. These type of compounds have been found to inactivate monoamine oxidase.[21]

Similar reactions can be postulated for pyrophosphate 20; in this case, irreversible inhibition would be achieved by thiomethyl elimination to give structures 28 or 29.



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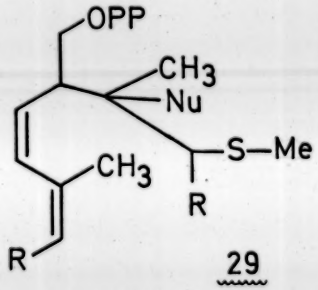

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Since proton removal must occur at some point in squalene synthesis, the approach suggested above will apply to any mechanism, provided that reactions prior to proton removal occur normally and the interfering chemical reaction competes effectively with the normal process following proton removal.

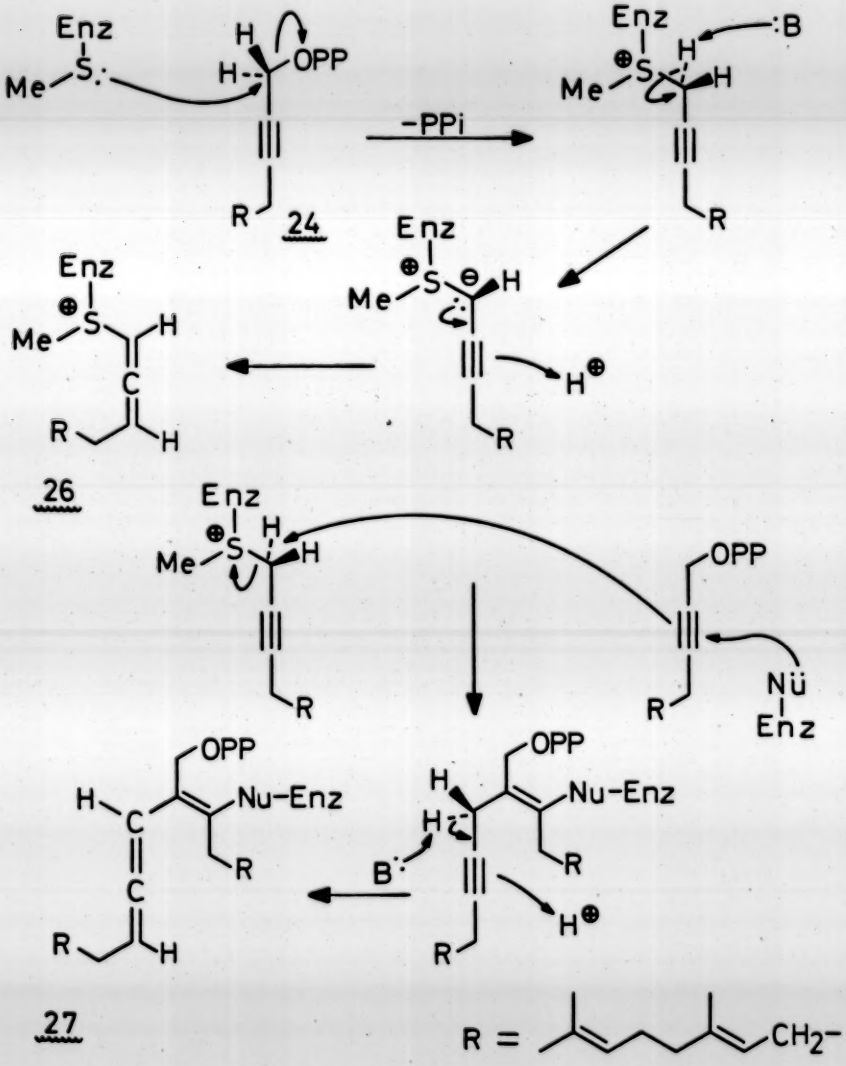

Fig.11

PART A

STUDIES OF SQUALENE SYNTHETASE INHIBITION

BY FARNESYL PYROPHOSPHATE ANALOGS

1. Source of Alcohol Precursors

a. E,E-Farnesol

E,E-farnesol was obtained by spinning band distillation of a commercial sample. Radioactive E,E-farnesol was prepared by reducing E,E-farnesal or E,E-methyl farnesoate with tritium labeled lithium aluminum hydride (table 4).[22]

It was observed that spinning band distilled E,E-ethyl farnesoate, prepared by modified Wittig-condensation of E-geranylacetone with triethyl phosphonoacetate-sodium hydride[22] (ratio 1: 1.3: 1 respectively), gives, after reduction with lithium aluminum hydride, about 15% of a high boiling impurity. The crude reduction mixture, however, could be separated by slow bulb-to-bulb distillation. The purified alcohol was oxidized with activated manganese dioxide to E,E-farnesal, the desired precursor.

b. Geraniol and Phytol

Geraniol and phytol, the precursors of 21 and 23, respectively, were purchased commercially. Phytol consisted of a 7:3 mixture of 2E and 2Z isomers.

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(22) R. H. Cornforth and G. Popják, Methods in Enzymol., 15, 359 (1969).

c. 3-Desmethylfarnesol (31); 7,11-Dimethyl-3-iodo-2(E),6(E),10-dodecatrien-1-ol (32); 7,11-Dimethyl-2-iodo-2(E),6(E),10-dodecatrien-1-ol (33); and 7,11-Dimethyldodeca-6(E),10-dien-2-yn-1-ol (30).

The farnesol analogs required in the synthesis of 16, 18, 19, and 24 were prepared according to the sequence outlined in Fig. 12. Reduction of 7,11-dimethyldodeca-6(E),10-dien-2-yn-1-ol 30[23] in THF using 1.3 equivalents of lithium aluminum hydride for 3 hr at room temperature afforded 3-desmethylfarnesol 31 in 92% yield.[24] The 3-iodo alcohol 32 has been reported to be produced stereospecifically by reaction of 30 with a solution of lithium aluminum hydride in THF using sodium methoxide as catalyst, followed by addition of excess iodine at -78°.[23a]

The 2-iodo alcohol 33, on the other hand, was reportedly formed when aluminum trichloride was used as catalyst in an analogous sequence.[23a] In the hands of this author, however, both catalysts produced the 3-iodo alcohol when combined with powdered lithium aluminum hydride. Alcohol 33 was therefore synthesized by a different procedure,[25] in which the alkoxide formed by addition of n-butyllithium to

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(23) (a) E. J. Corey, J. A. Katzenellenbogen, and G. H. Posner, J. Am. Chem. Soc., 89, 4245 (1967); (b) P. R. Ortiz de Montellano, Ph. D. Thesis, Harvard University, 1968, p 123.

(24) (a) 23; (b) B. Grant and C. Djerassi, J. Org. Chem., 39, 968 (1974).

(25) E. J. Corey, H. A. Kirst, and J. A. Katzenellenbogen, J. Am. Chem. Soc., 92, 6314 (1970).

a solution of 30 in ether is treated with diisobutylaluminum hydride and excess iodine. A distinction between the two iodo compounds was possible on the basis of the nmr shifts of the C-1 protons (nmr 1,2). In the 3-iodo alcohol 32, the methylene protons appear as a doublet at 4.11ppm due to coupling with the vinyl proton at C-2, which appears as a triplet at 5.73ppm. In the 2-iodo compound 33, no coupling is possible and the C-1 protons appear as a singlet at 4.24ppm.

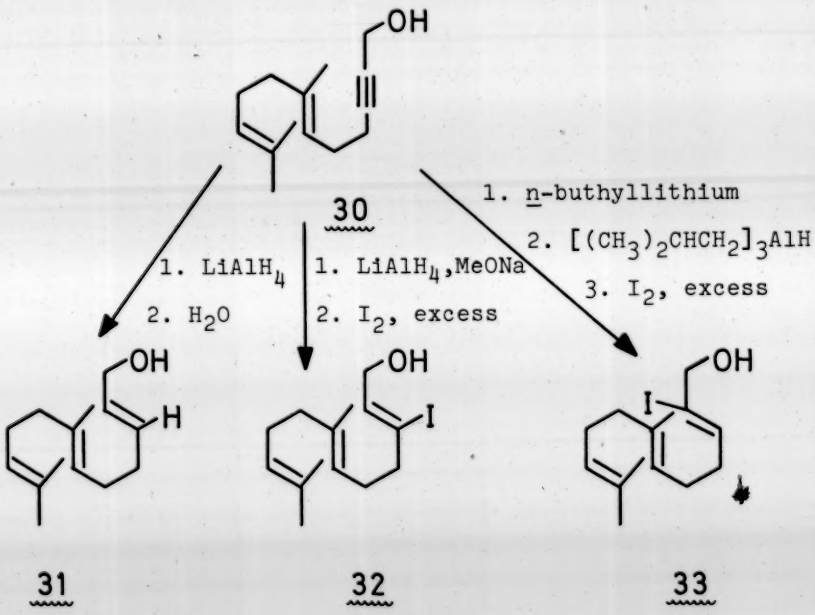

Fig.12

d. Hexahydrofarnesol (34)

Preparation of hexahydrofarnesol (34) by catalytic hydrogenation of farnesol in the presence of 15% palladium-on-charcoal catalyst led to hydrogenolysis products with very low yields of the desired compound.[26] Hydrogenation of a stereoisomeric mixture of ethyl farnesoate, on the other hand, gave a single compound in 96% yield. The saturated ester 35 was reduced to 34 with lithium aluminum hydride in 92% yield (nmr No. 3).

e. 2-Methylfarnesol (36)

The synthesis of 2-methylfarnesol (36) is presented in Fig. 13. Reaction of triethylphosphite with α-bromopropionoate afforded diethyl 1-carboethoxyethylphosphonate.[27] Wadsworth—Emmons condensation[28] of this ester with E-geranylacetone[29] gave a 1:1 mixture of 2-E and 2-Z isomers of ethyl 2-methylfarnesoate (37) in 84% yield. The isomeric mixture was separated by spinning band distillation and the 2-E isomer reduced with lithium aluminum hydride to furnish 2E 36 (nmr No. 6), in 95 % yield. Popják et al.,[30]

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(26) E. D. McCarthy and M. Calvin, Tetrahedron, 23, 2609 (1967).

(27) L. J. Dolby and G. N. Riddle, J. Org. Chem., 32, 3481 (1967).

(28) W. S. Wadsworth and W. D. Emmons, J. Am. Chem. Soc., 83, 1733 (1961).

(29) (a) O. Isler, R. Ruegg, L. Chopard-dit-Jean, H. Wagner,and K. Bernhard, Helv. Chim. Acta, 39, 897 (1956); (b) I. N. Nazarov, S. M. Makin, O. A. Shavrygin, and V. A. Smirnyagin, J. Gen. Chem., USSR, 30, 467 (1960).

(30) G. Popják, P. W. Holloway, and J. M. Baron, Biochem. J., 111, 325 (1969).

have reported that all -E isomers of prenyl alcohols and esters have higher retention times (tr) on glc than isomers with one or more double bonds in the Z-configuration. This observation has been confirmed and extended in this work (table 1).

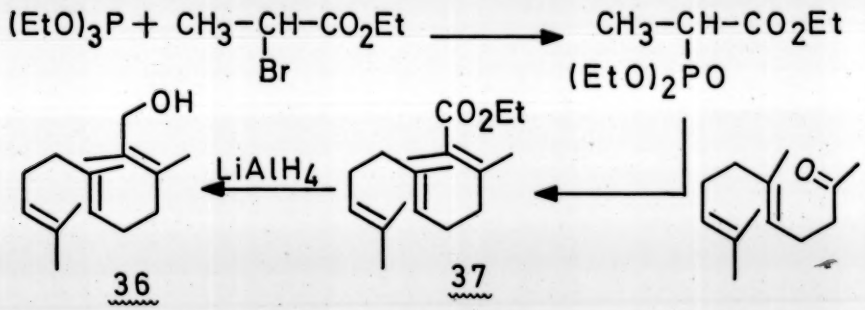


Fig.13

The assignment of stereochemistry to 2-methylfarnesol, suggested by its relative retention time, was independently verified using the ethyl ester precursor 37 (nmr 4,5). Analysis of the nmr data in Fig. 14 shows a close correlation between the shifts of the C-2, and particularly C-3, methyl groups in the longer retention time isomer with the corresponding methyls in methyl-2,3-dimethyl-2-butenoate.[31]

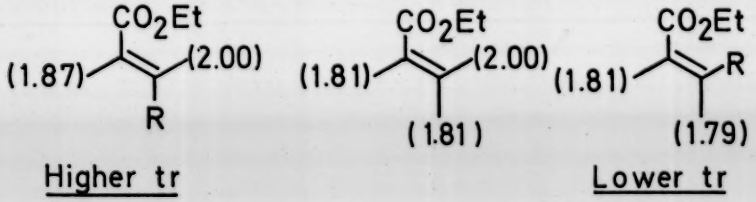


Fig.14

(31) T. V. Van Auken and K. L. Rinehart, J. Am. Chem. Soc., 84, 3736 (1962).

Table 1. Physical Properties of Precursors

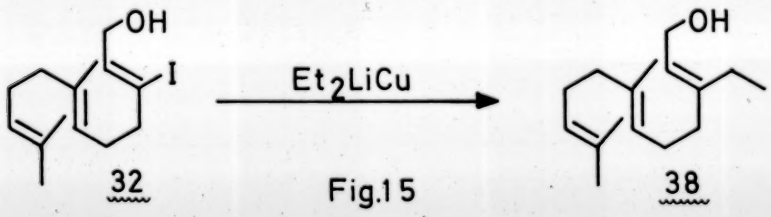
Alcohol Precursor	GC Retention Time (min)[a] 2Z	2E	Temp(°C)	Rf[b] 2-Z	2-E
Farnesol	13.13	15.03	150	0.32	0.28
2-Methyl (36)	17.40	20.29	150	0.37	0.29
3-Desmethyl (31)	9.26	10.05	150		0.29
3-Ethyl (38)	18.75	20.36	150	0.35	0.31
3-Iodo (32)		20.93	175		0.40
2-Iodo (33)		21.94	175		0.44
4-Thiomethyl		24.94	175		0.25
Geraniol	4.39	4.91	125		0.23
Tetrahydro	18.06	9.19	150		0.30
Phytol	13.58	15.56	175		0.35
Propargylic (30)	15.03		150		0.37
Hexahydro (34)	7.01		150		0.30

Ethyl Ester Precursor				Rf[c] 2-Z	2-E
Farnesoate	13.00	17.00	150	0.49	0.40
2-Methyl (37)	17.00	21.00	150	0.44	0.31
3-Ethyl (40)	18.00	20.80	150	0	0.30
4-Thiomethyl		8.5	175	0.47	0.42
Tetrahydro	7.5	11.1	150	0.58	0.47
Hexahydro (35)	6.11		150	0.49	

[a]System A. [b]Benzene-ethyl acetate, 10:1. [c]3% Ethyl acetate in hexane, 2 developments.

f. 7,11-Dimethyl-3-ethyl-2(E),6(E),10-dodecatrien-1-ol (38).

One method for carbon-carbon bond formation between unlike groups is the selective coupling of n-alkyl copper reagents with vinyl halides.[32] Application of this procedure to the synthesis of 38 was attempted (Fig. 15).



32 Fig.15 38

Reaction of 32 with 4 mole equivalents of lithium diethyl copper in ether at -20° for 2 hr, followed by treatment with excess ethyl iodide at 0° for 18 hr, gave 56% of a mixture containing alcohol 31, 2 Z 38, and 2 E 38 in a ratio 1.5: 1: 10 by glc. This mixture was not separated since the components are essentially superimposed on tlc (table 1). Contamination of 38 with even traces of 3-desmethylfarnesol could not be tolerated, since the contaminant might itself prove active. A different approach for the synthesis of 38 was therefore undertaken (Fig. 16).

Reaction of ethyl-3-ketopentanoate (sodium salt) with geranyl bromide,[33] followed by basic hydrolysis and

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(32) (a) E. J. Corey and G. H. Posner, J. Am. Chem. Soc., 90, 5615 (1968); (b) E. J. Corey and G. H. Posner, ibid., 89, 3911 (1967); (c) 21a

(33) P. R. Ortiz de Montellano, Ph. D. Thesis, Harvard University, 1968, p 75.

decarboxylation, gave 1-methylgeranylacetone (39). Modified Wittig-condensation[28] of 39 with triethyl phosphonoacetate yielded a 3:2 mixture of 2 Z and 2 E isomers of ethyl ester 40. Spinning band distillation and ester reduction of 2 E isomer (nmr 9) with sodium bis- (methoxyethoxy) aluminum hydride[34] gave 38.

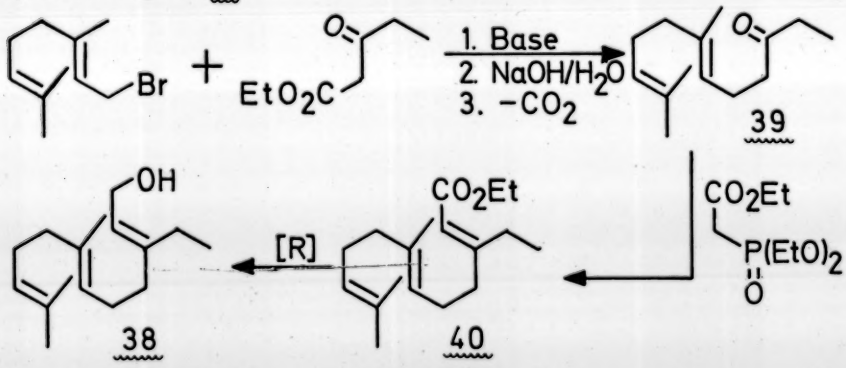

Fig.16

Unambiguous assignment of the stereochemistry of 38 could not be made by nmr correlation with model structures.[35] Differentiation of the geometrical isomers was accomplished by nmr studies using the shift reagent $Eu(fod)_3$.[36] A series of nmr spectra (No. 7 and 8) was recorded for each isomer with increasing concentrations of the shift reagent. The resulting effect on the 3-ethyl protons is presented in

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(34) M. Černý, J. Málek, M. Čapka and V. Chvalovský, Coll. Czech. Chem. Commun., 34, 1025 (1968).

(35) K. Ogura, T. Nishino, T. Koyama and S. Seto, J. Am. Chem. Soc., 92 6036 (1970).

(36) R. E. Rondeau and R. E. Sievers, ibid., 93, 1522 (1971).

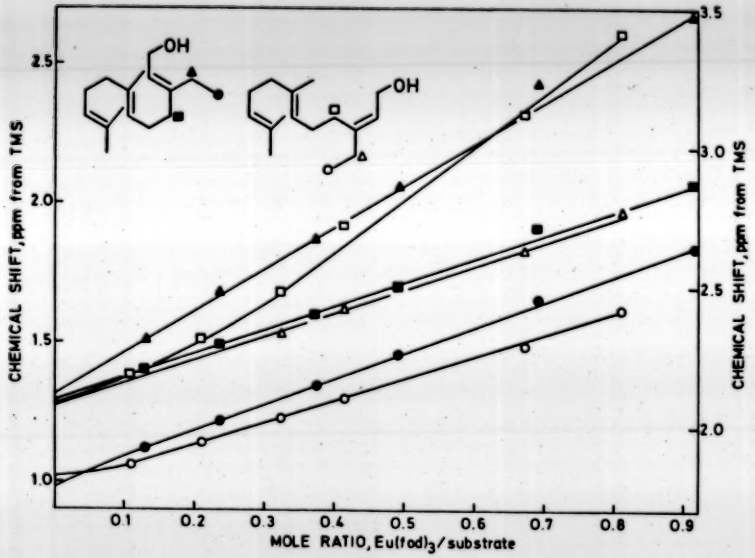

Figure 17. Variation in the chemical shift of some of the protons in 3-ethyl alcohol 38 upon addition of $Eu(fod)_3$ in $CDCl_3$ (left-hand ordinate for the methyl protons, right-hand ordinate for the methylene protons).

Fig. 17. Clearly, as the ratio of shift reagent to analog increases, a larger shift is observed for the 3-ethyl protons in the isomer with higher retention time. This isomer, assigned 2 E stereochemistry,[37] had identical Rf and tr as the major compound from the former reaction.

All the farnesol analogs, except phytol, were essentially pure all-E isomers, with less than 4% of the 2 Z isomers present as judged by glc analysis.

2. Synthesis of Pyrophosphates

A general procedure for the synthesis of pyrophosphates, in which high yields are obtained, is still unavailable. The original method of Cramer and Bohm,[38] as modified by Popják et al.,[3c,9e,22] involves treatment of prenyl alcohol with excess di-triethylammonium phosphate and trichloroacetonitrile. The pyrophosphates, contaminated with monophosphates and unreacted phosphorylating agent, are extracted with aqueous ammonia. Separation of the organic material is based on the lipophilic character of the farnesyl chain which leads to selective adsorption on Amberlite XAD-2 resin, while inorganic materials remain in the aqueous ammonia. The organic phosphates are then eluted and separated on a DEAE cellulose column. Finally, adsorption on Amberlite

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(37) R. F. Cockerill, G. L. O. Davies, R. C. Harden, and D. M. Rockham, Chem. Rev., 73, 553 (1973).

(38) F. Cramer and W. Bohm, Angew. Chem., 71, 775 (1959).

XAD-2 resin, as before, gives the purified pyrophosphates.[9e,22]

Although this method gives essentially pure pyrophosphates, it is very long and tedious, particularly when working with radioactive materials. A simplified purification procedure was therefore developed.

The analogs of farnesyl pyrophosphate were synthesized using the procedure described above. The crude reaction mixture was not extracted, however, since difficultly separable emulsions are formed. Instead it was concentrated to a small volume and all phosphates were precipitated by addition of concentrated ammonia. The inorganic salts were removed as before by adsorption on XAD-2 resin. Selective precipitation of pyrophosphates was achieved by addition of acetone containing a few drops of concentrated ammonia to the resulting mixture of organic phosphates. The crude reaction mixture can also be separated by column chromatography on silica gel with excellent results.[18,39] Although tlc analysis showed a single spot for the pyrophosphates, quantitative phosphorus determinations (table 2)[40] showed apparent traces of mono or polyphosphates in some cases.

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(39) M. J. Kessler and J. M. Clark, Analyt. Biochem., 35, 160 (1973).

(40) G. R. Bartlett, J. Biol. Chem., 234, 466 (1959).

Table 2. Unlabeled Pyrophosphates

Compound	Yield (%)	Phosphorus analysis (% of theory)	Monophosphate Rf[a]	Pyrophosphate Rf[a]
1	20	101	0.55	0.34
15	17	114	0.55	0.34
16	33	106	0.55	0.34
17	16	93	0.57	0.43
18	23	100	0.55	0.34
19	10	93	0.55	0.34
20	9	103	0.42	0.21
21	22	102		
22		107		
23	16	95		
24	11	91	0.57	0.41
25	9	93	0.59	0.43

[a]Solvent: n-propanol-conc. NH_4OH-H_2O =6:3:1

3. Alkaline Phosphatase Hydrolysis.[41]

The organic pyrophosphates were hydrolyzed by incubation with bacterial alkaline phosphatase.[7] The incubation mixture was extracted with hexane or petroleum ether and the extracts concentrated *in vacuo*. The recovered alcohols were compared with authentic samples by tlc and glc. In each case the recovered alcohol had essentially the same Rf and tr as the starting material (table 1).

4. Enzyme Preparation.

Yeast Squalene Synthetase was prepared by a method of Qureshi et al.,[12] as modified by Dr. J. S. Wei in these laboratories.[18]

5. Inhibition Studies.

The relative potencies of the synthetic analogs as inhibitors were determined by measuring the decrease in enzymatic incorporation of [1-^{3}H] -farnesyl pyrophosphate into squalene caused by fixed concentrations (0-6 μM) of the analogs. The details of the procedure, developed by Dr. J. S. Wei, are described in Ref. 18.

The results of these studies are presented in Fig. 18 as plots of percent inhibition, relative to control incubations with no inhibitor, *versus* the concentration of the inhibitor used. It is seen that the highest inhibition was obtained with 15 and 17, while the weakest inhibition

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(41) Part of the biological studies were done in cooperation with Dr. J. S. Wei. Preliminary studies with Dr. S. M. Byun.

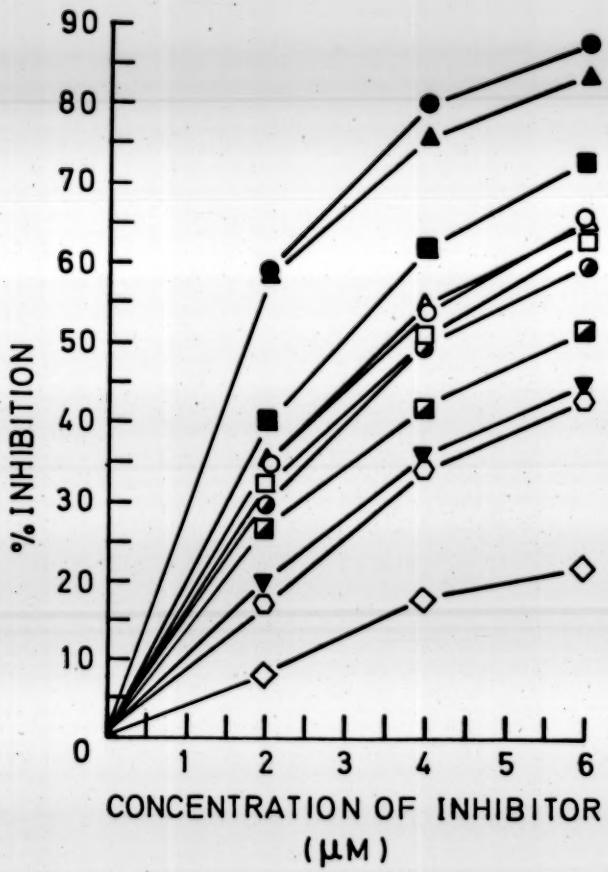

Figure 18. Relative inhibition of squalene synthetase by farnesyl pyrophosphate analogs. The incorporation of [1-^{3}H]-farnesyl pyrophosphate (2.5 μM) into squalene in the presence of 2,4, and 6 μM concentrations of inhibitor is plotted as percent of the incorporation observed with no inhibitor present. Inhibitor: 15(●), 17(▲), 16(■), 20(○), 18(△), 19(□), 24(◕), 23(◪), 22(▼), 25(⬭), and 21(◇).

resulted with geranyl pyrophosphate (21). Phytyl pyrophosphate 23 is slightly more potent than shown, since no adjustment was made for the presumably less active 2 Z isomer.

Some of the alcohol precursors themselves were similarly evaluated as inhibitors. None of these gave significant inhibition when present in concentrations up to 20 μM. Parallel results were obtained by A. S. Boparai with (22) monophosphate, which causes only 6% inhibition in 6 μM concentration.

6. Kinetic Analysis of the Inhibition

In order to determine if the inhibitors compete with the normal substrate for the active site of the enzyme, a series of incubations were carried out by Dr. J. S. Wei to obtain data for kinetic analysis. Double reciprocal plots of initial reaction velocities versus substrate concentration, at fixed inhibitor concentrations, were made for each analog. The results are illustrated in Fig. 19-29 and are summarized in table 3. It was not possible to calculate accurately the inhibition constants due to the relatively crude enzyme system employed. The farnesyl pyrophosphate Km value, for example, varied slightly from one enzyme preparation to another. Nevertheless, 15 was found to have a Ki of about 0.5 μM. The inhibition patterns show that each analog is a competitive, or at least partially competitive (mixed), inhibitor.[42]

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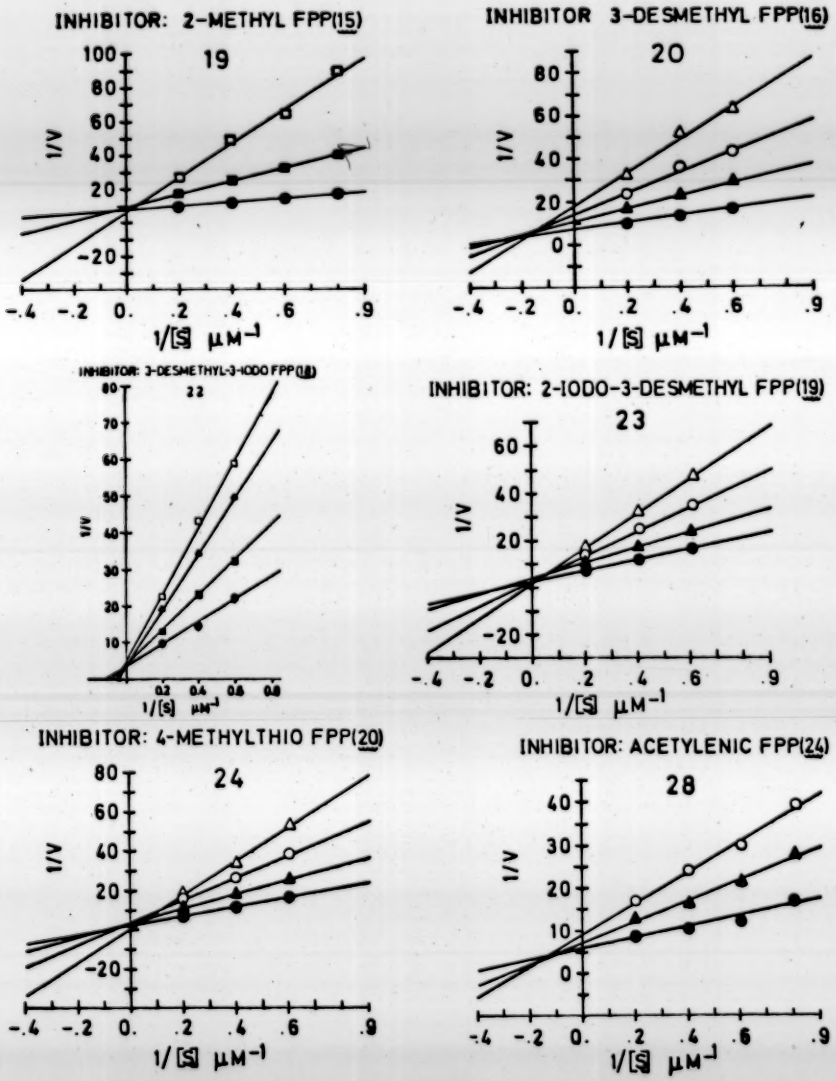
(42) J. L. Webb, "Enzyme and Metabolic Inhibitors", Vol. 1, Academic Press, New York, N.Y., 1963, p. 157-180.



Figures 19, 20, 22, 23, 24, and 28. Lineweaver-Burk graphic analyses of the inhibition of squalene synthetase by farnesyl pyrophosphate analogs 15, 16, 18, 19, 20, and 24. [S] is the concentration of [1-^{3}H]-farnesyl pyrophosphate; initial velocity (V) is expressed in nanomoles of squalene formed per minute per milligram of protein. Concentration of inhibitor: 0(●), 1μM(■), 2μM(▲), 3μM(□), 4μM(○), and 6μM(△).

An equivalent computerized graphic analysis is not yet available for the rest of the analogs. Nonetheless the observed inhibition values are as follows:

For figure 21. Inhibitor:3-Ethyl analog 17

$1/[S]\mu M^{-1}$	1/V at 17 μM: 0	2	4	6
0.2	3.49	4.59	5.78	7.69
0.3	4.08	5.76	8.06	10.66
0.4	5.03	7.80	10.46	13.49
0.6	7.12	10.73	14.90	20.43

For figure 25. Inhibitor:Geranyl PP 21

$1/[S]\mu M^{-1}$	1/V at 21 μM: 0	2	4	6
0.2	2.55	2.96	3.33	3.58
0.4	3.95	4.26	4.83	5.08
0.6	5.30	6.09	6.58	7.12
0.8	7.55	8.27	9.02	9.94

For figure 26. Inhibitor:Tetrahydro FPP 22

$1/[S]\mu M^{-1}$	1/V at 22 μM: 0	2
0.2	3.51	4.16
0.4	5.74	7.04
0.6	8.45	10.87
0.8	11.14	15.60

For figure 27. Inhibitor:Phytyl PP 23

$1/[S]\mu M^{-1}$	1/V at 23 μM: 0	2	4	6
0.1600	3.33	4.19	4.99	5.84
0.2000	4.07	5.32	6.25	7.09
0.2666	5.38	6.83	8.28	9.37

For figure 29. Inhibitor:Hexahydro FPP 25

$1/[S]\mu M^{-1}$	1/V at 25 μM: 0	2	4
0.1600	2.33	2.46	2.86
0.2000	2.37	2.71	3.38
0.2666	2.57	3.35	4.12
0.4000	3.50	4.21	5.53

Table 3 Classification of Inhibitors

15 competitive	19 competitive	23 competitive
16 mixed	20 competitive	24 mixed
17 competitive	21 competitive[1]	25 competitive
18 competitive	22 competitive[2]	

(1) The lines due to this weak inhibitor did not differ sufficiently in slope for unambiguous assignment.

(2) The lines in the Lineweaver-Burk plot curved upwards at values of $1/[s]$ greater than 0.6 μM^{-1}.

Rearrangement of analog 16 to 3-desmethylnerolidyl pyrophosphate was considered a possible explanation for the mixed inhibition observed with 16. This possibility was ruled out when 16 was hydrolyzed with alkaline phosphatase and the hexane extractable product compared with authentic 31 and synthetic 3-desmethylnerolidol[43] by tlc and glc. Only 3-desmethylfarnesol (31) was detected.

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(43) This compound was prepared by the sequence in Fig. 30. Oxidation of alcohol 41 (ref. 29b) with pyridinium chlorochromate in dichloromethane, buffered with sodium acetate (ref. 44), afforded aldehyde 42 in 49% yield. Reaction of tetravinyl tin with 3 equivalents of n-butyllithium (ref. 45), followed by addition of aldehyde 42 yielded 79% of 3-desmethyl nerolidol 43 (ref. 46). The nmr (No. 10) is reproduced in this thesis.

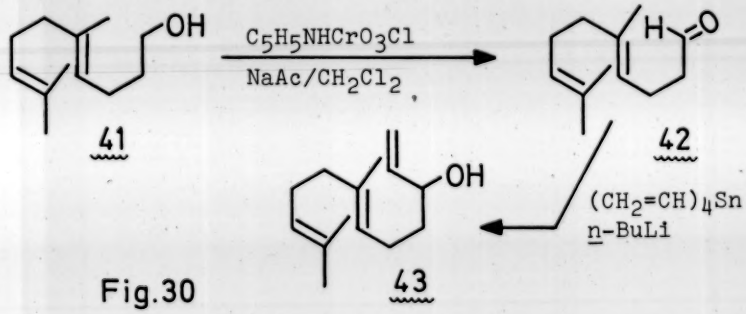

Fig.30

(44) E. J. Corey and J. Williams Suggs, Tetrahedron Lett., 2647 (1975)

(45) D. Seyferth and M. A. Weiner, J. Am. Chem. Soc., 83, 3583 (1961).

(46) O. P. Vig, R. C. Anand, G. L. Kad and J. M. Sehgal, J. Indian Chem. Soc., 47, 999 (1970).

7. Preincubation Studies

The kinetic mechanism of Beytia et al.,[13] discussed before, involves a probably covalent farnesyl-enzyme intermediate. It is probable that suitably modified farnesyl pyrophosphate molecules would be accepted as substrates, forming the covalent complex, but would then be unable to complete the synthetic sequence, resulting in irreversible inhibition. Compounds 16, 18, 19, 20, and 24, in particular, were prepared with such a possibility in mind. To determine if irreversible inhibition was occurring the analogs were preincubated for up to five hours at 25° with the enzyme and all assay components, except the usual substrate. Normal bioassay was then initiated by addition of [1-^{3}H]-1. Irreversible binding is characterized by a time-dependent increase in observed inhibition.[47] When the percent inhibition was plotted versus preincubation time, Fig. 31 was obtained. The time-dependent decrease in observed inhibition is incompatible with irreversible binding. The decrease in inhibition with time may be due to slow enzymic and chemical degradation of the inhibitors. The validity of these studies rest on the reasonable but not inviolate assumption that irreversible inhibition competes with degradation of the substrates.

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(47) W. N. Aldridge and E. Reiner, Enzyme Inhibitors as Substrates, American Elsevier, New York, N.Y., 1972.

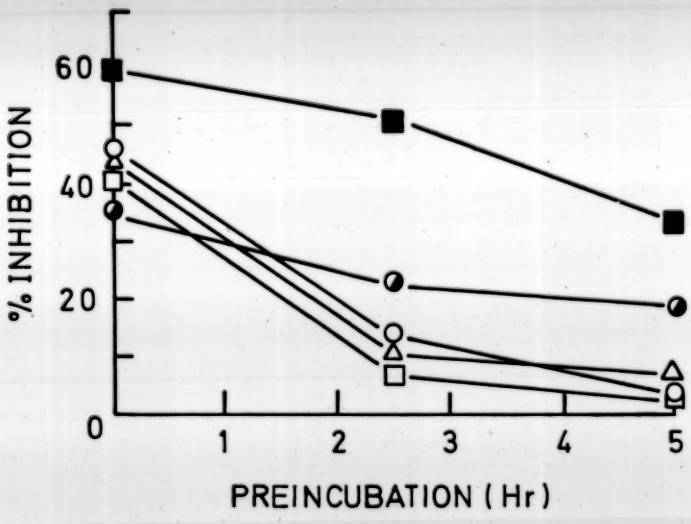

Figure 30. Effect of incubating inhibitors (4 μM) with squalene synthetase prior to addition of [1-^{3}H]-farnesyl pyrophosphate (5 μM) and normal bioassay. Preincubation was at ambient temperature with the full assay system, excluding the substrate. Inhibitor: 16(■), 18(△), 19(□), 20(○), and 24(◐).

PART B

ARTIFICIAL SUBSTRATES FOR SQUALENE SYNTHETASE

1. Introduction

This section deals with a study of the enzyme mechanism and active site topology using farnesyl pyrophosphate analogs as pseudo substrate probes. Specifically, tritium labeled analogs 15, 16, 17, 20, and 22 have been tested as artificial substrates for squalene synthetase. Although part of our results are already available,[48] the details of these studies are presented here.

Analogs of farnesyl pyrophosphate, such as those discussed in the first part of this work, can be of value for the investigation of the properties of squalene synthetase. These analogs bear modifications primarily around the C-2 double bond, an area which is directly involved in the catalytic process at the active site. It is therefore of interest to determine the influence of steric as well as electronic effects due to substituents on the course of the condensation.

Previous studies on the specificity of this condensation have been limited to the use of artificial substrates,

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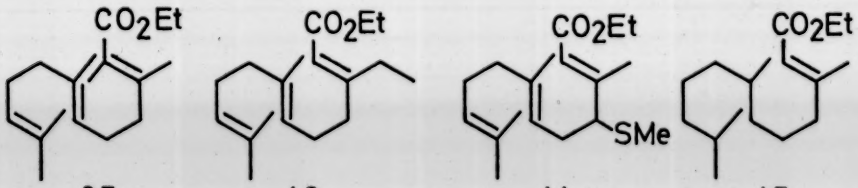
(48) (a) P. R. Ortiz de Montellano, R. Castillo, W. Vinson, and J. S. Wei, J. Am. Chem. Soc., 98, 3020 (1976); (b) P. R. Ortiz de Montellano, R. Castillo, W. Vinson, and J. S. Wei, ibid; 98, 2018 (1976).

analogs of farnesyl pyrophosphate, which have an elongation at the C-12 position.[49] These studies have shown that increasing the length of the chain by one methyl group is tolerated by the enzyme from liver homogenates.[49a,b] The observed products from these condensations are squalene analogs in which the terminal positions are extended by methyl groups; however, formation of squalene analogs with ethyl groups at these positions was not observed.[49b] In contrast, purified yeast squalene synthetase was shown to condense a longer analog, geranylgeranyl pyrophosphate, to lycopersene, a C_{40} squalene analog.[49c] It is possible that ethyl squalene analogs were not observed with the liver system either because of low sensitivity during detection, or perhaps because of differences in the enzyme systems used.

2. Precursors

a. Ethyl esters

The synthesis of 2-E ethyl ester precursors 37, 40, 44, and 45, has been discussed in the previous section. Analysis by glc showed that these precursors were essential-



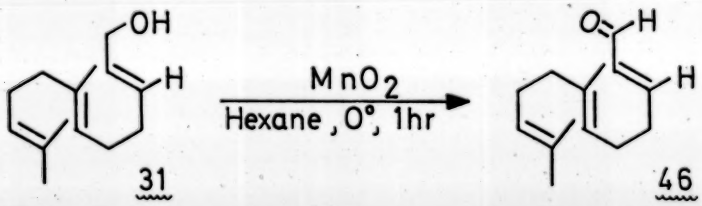
37 40 44 45

(49) (a) A. Polito, G. Popjak, and T. Parker, J. Biol. Chem., 247, 3464 (1972); (b) K. Ogura, T. Koyama, and S. Seto, J. Am. Chem. Soc., 94, 307 (1972); (c) A. A. Qureshi, F. J. Barnes, E. J. Semmler, and J. W. Porter, J. Biol. Chem., 248, 2755 (1973).

ly all-E isomers, contaminated in no instance with more than 4% of the 2 Z isomer.

b. 3-Desmethylfarnesal (46)

This compound was readily obtained in 67% yield by oxidation of (2-E)-3-desmethylfarnesol 31 with activated manganese dioxide.[50]



31 46

Oxidations of this type have been reported to proceed with no isomerization of the allylic double bond.[50] In this case only one peak was detected by glc. The infrared spectrum (No. 1) showed a strong band at 5.87μ while the nmr spectrum (No. 11) was identical to that of 46 prepared by a different procedure.[51]

c. Tritium Labeled Alcohols

Popják et al.,[3c] have reported the preparation of tritium labeled farnesol by reduction of methyl farnesoate with 1.5 equivalents of radioactive lithium aluminum hydride in ether at - 30° for 90 min.

The synthesis of the labeled alcohol precursors of the pyrophosphate analogs was carried out by a related procedure. Ethyl esters 37, 40, and 44, were treated with a

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(50) E. J. Corey, N. W. Gilman, and B. E. Ganeni, J. Am. Chem. Soc., 90, 5616 (1968).
(51) A. F. Thomas, ibid., 91, 3281 (1969)

fraction of an equivalent of unlabeled lithium aluminum hydride under strictly anhydrous conditions, then with tritium labeled lithium aluminum hydride, and finally again with unlabeled lithium aluminum hydride. The first fraction of hydride added was such that enough ester would remain to incorporate all the labeled material. In the case of alcohol 31, the reduction was carried out on the aldehyde (46) instead of the ester. The results of these preparations are in table No. 4. In each case the labeled alcohol cochromatographed with an authentic sample of unlabeled material.

3. Synthesis of Tritium Labeled Pyrophosphates

The synthesis of analogs 15, 16, 17, and 20 was carried out exactly as described for the preparation of unlabeled pyrophosphates. The results are in table 4. The structure of these labeled pyrophosphates was confirmed by quantitative phosphorus analysis (table 4).[40]

4. Incubations

Standard incubations of the analogs with an insoluble yeast enzyme preparation were carried out by J. S. Wei in these laboratories.[49] Incubations (37°, 1-10 min) were terminated by addition of ethanol (ethanol/volume of incubation, 2/1) and the hydrocarbon products, extracted with light petroleum ether, were chromatographed on silica gel or neutral alumina before liquid scintillation counting. These results are listed in table 5.

Table 4. Preparation of Tritium Labeled Alcohols and Pyrophosphates.

Precursor (mmol)	Sequence of Hydrides (mmol)	Alcohol obtained (% yield)	Incorporation of label %	Specific activity mC/mM	Pyrophosphate % Yield	Specific Activity mC/mM	Phosphorus analysis (% of theory)
Methyl ester (0.9218)	0.1320 0.1163(5mC) 0.3680	Farnesol (92)	67	4	17	0.37	100
Aldehyde (0.6376)	0.1425 0.0375(5mC) 0.0264	Farnesol (100)	38	3	12	5.86	98
Ethyl ester (0.3772)	0.0792 0.0369(5mC) 0.0792	2-Methyl-farnesol (98)			15	0.5	97
Aldehyde (0.4850)	0.037 0.037 (5mC) 0.053	3-Desmethyl farnesol (95)			13	16.22	87
Ethyl ester (0.5000)	0.0193 0.0380(5mC) 0.0450	3-Ethyl analog 38 (95)	5	0.5	9	0.99	95
Ethyl ester		4-Thiomethyl farnesol			8	4.95	95

Table 5. Conversion of Farnesyl Pyrophosphate Analogs to Hydrocarbons.

Starting substrate	(10^3dpm)	(nM)	Net incorporation into hydrocarbon (10^3dpm)	(nM)	Control with inactive enzyme (dpm)	Incorporation relative to 1(%)
1	(200)	(15)	(13.051)	(0.979)	74	100
15	(17)	(15)	(0.069)	(0.061)	76	6.23
16	(539)	(15)	(0.268)	(0.007)	203	0.71
17	(33)	(15)	(0.004)	(0.002)	88	Statistically Insignificant
20	(166)	(15)	(0.018)	(0.002)	89	"
22	(526)	(15)	(0.074)	(0.002)	261	"

Only analogs 15 and 16 gave GC detectable radioactive hydrocarbon products. These behaved like squalene on silica gel tlc. Incorporation of the analogs into products was absolutely dependent on the presence of NADPH and active enzyme (Fig. 32, 33), while it was stimulated by addition of 1 to the incubation mixture (Fig. 34, 35). Incorporation of [1-^{3}H]-15 relative to that of [1-^{3}H]-1 (in a standard 1ml, 1 min incubation) amounted approximately to 6.23%, depending on the enzyme preparation. Incorporation of [1-^{3}H]-16 in analogous standard incubations varied from 0.25 to 1%.

5. Identification of Hydrocarbon Products

The most likely structures for the radiolabeled products obtained from incubations of 15 and 16 were either symmetrical squalene analogs derived from two units of the same substrate (47 or 48), or unsymmetrical squalene analogs (49 or 50) due to mixed condensation between the normal substrate 1 and the analog. This reasoning is based on three facts: (1) the need for active enzyme implies

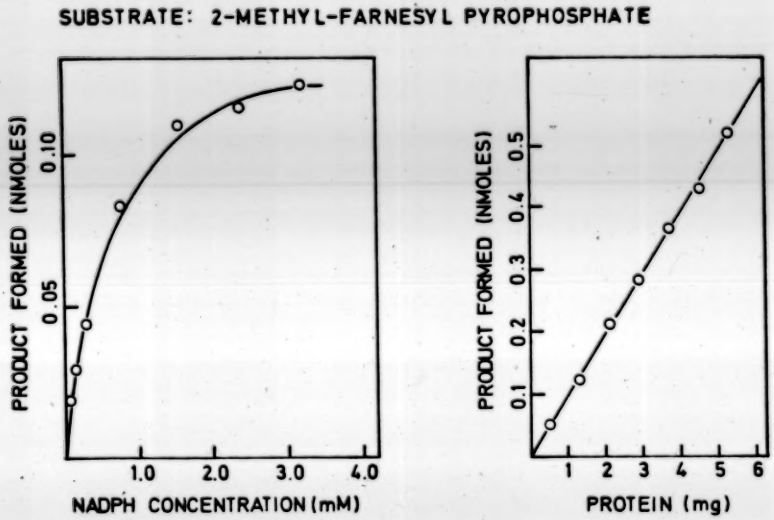


Figure 32. Effect of NADPH concentration and active enzyme on the formation of labeled hydrocarbon product obtained by a 10 min incubation of [1-^{3}H]-2-methylfarnesyl pyrophosphate with a standard mixture.[18]

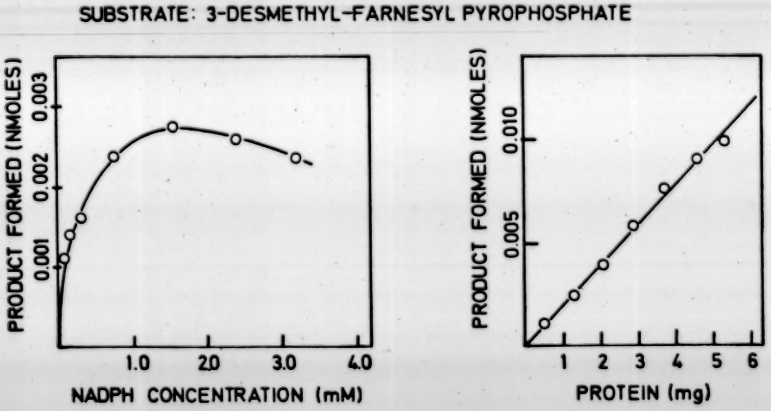


Figure 33. Effect of NADPH concentration and active enzyme on the formation of labeled hydrocarbon product obtained by a 3 min incubation of [1-^{3}H]-3-desmethylfarnesyl pyrophosphate with a standard mixture.[18]

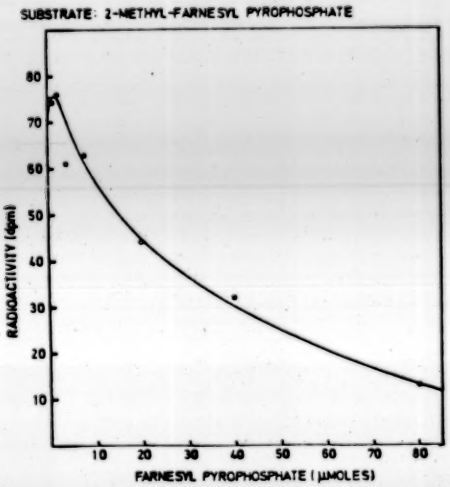


Figure 34. Effect of FPP concentration on the formation of labeled hydrocarbon product obtained by a 10 min incubation of [1-^{3}H]-2-methylfarnesyl pyrophosphate with a standard mixture.[18]

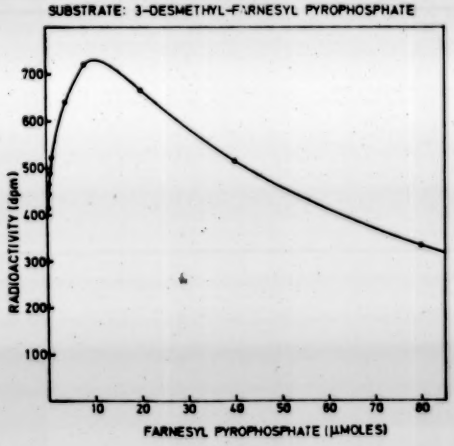
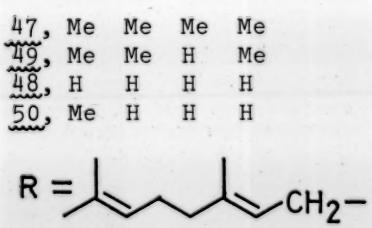
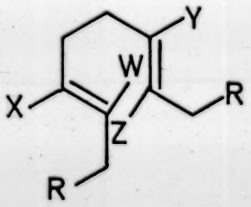


Figure 35. Effect of FPP concentration on the formation of labeled hydrocarbon product obtained by a 10 min incubation of [1-^{3}H]-3-desmethylfarnesyl pyrophosphate with a standard mixture.[18]

that the products are formed by enzymic action and not merely by chemical degradation of the substrates, (2) the requirement for NADPH is consistent with the cofactor dependency of squalene synthetase, and (3) enhancement of product formation by addition of farnesyl pyrophosphate, suggests the occurrence of a mixed condensation. The synthesis of these hydrocarbons was therefore undertaken to obtain authentic reference samples.

	W	X	Y	Z
47,	Me	Me	Me	Me
49,	Me	Me	H	Me
48,	H	H	H	H
50,	Me	H	H	H





a. Synthesis of 11,14-Dimethylsqualene (47)

The synthesis of this compound was carried out by two different procedures (Fig. 36). The first approach is based on the recently described stereospecific (but not regiospecific) coupling of allylic bromides by cuprous iodide-dialkylamide complexes.[52] With this procedure, for example, (E,E)-farnesyl bromide is converted to all-E squalene, while (Z,E)-farnesyl bromide produces (E,E,Z,Z,E,E)-squalene. Both condensations reportedly proceed with 98% stereospecificity. The other products observed in these reactions are regioisomers due to (1-3) and (3-3) conden-

(52) K. Kitagawa, K. Oshima, H. Yamamoto, and H. Nozaki, Tetrahedron Lett., 1859 (1975).

sation, among which the (1-3) is predominant.[52] The second procedure derives from the well known reaction of allylic bromides with nickel tetracarbonyl.[53] The products obtained in these nonstereospecific condensations are usually geometrical isomers in which the E,E-compound is predominant, irrespective of the stereochemistry of the starting material. The distribution of isomers, however, varies with the double bond substitution pattern. Coupling of geranyl bromide, for example, gives a product that was shown to consist of Z,Z-; Z,E-; and E,E- isomers in the ratio 1: 7: 8.[54]

On glc the Z,Z- isomer has the lowest and the E,E-isomer the highest retention time. On the other hand, coupling of 3-desmethylfarnesyl bromide also gave Z,Z-; Z,E-; and E,E- isomers, but in the ratio 1: 9: 90.[55] The relative retention times of these isomers were as before; e.g., highest for the E,E-isomer.[55]

2-Methylfarnesyl bromide (51) was readily obtained by stirring 2-methylfarnesol (36;2Z:2E,2:98) with phosphorus tribromide in ether. This well known reaction of allylic alcohols proceeds without appreciable isomerization of

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(53) For an excellent review on the synthetic value of this reagent see (a) M. F. Semmelhack, Org. Reactions, 19, 115 (1972); see also (b) R. Baker, Chem Rev., 73, 487 (1973); (c) W. Carruthers, Chem. Ind. (London), 931 (1973).

(54) M. F. Semmelhack, Ph. D. Thesis, Harvard University, 1967, p. 56.

(55) P. R. Ortiz de Montellano, Ph. D. Thesis, Harvard University, 1968, p. 126.

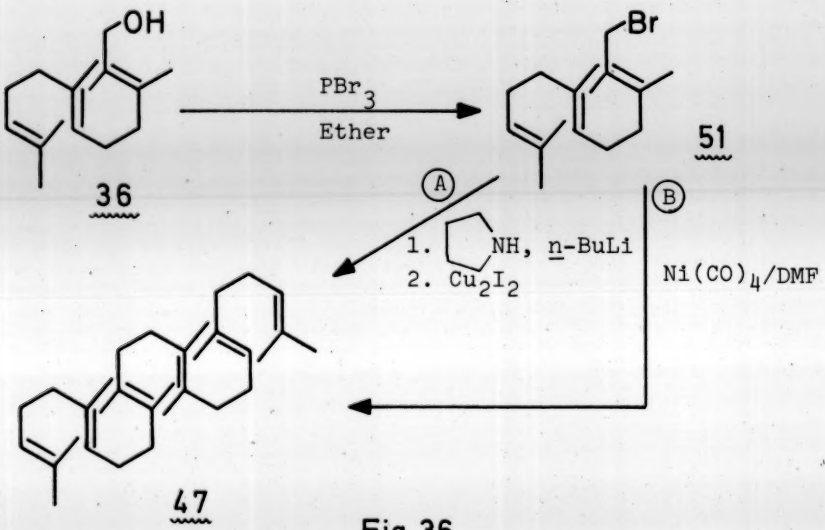
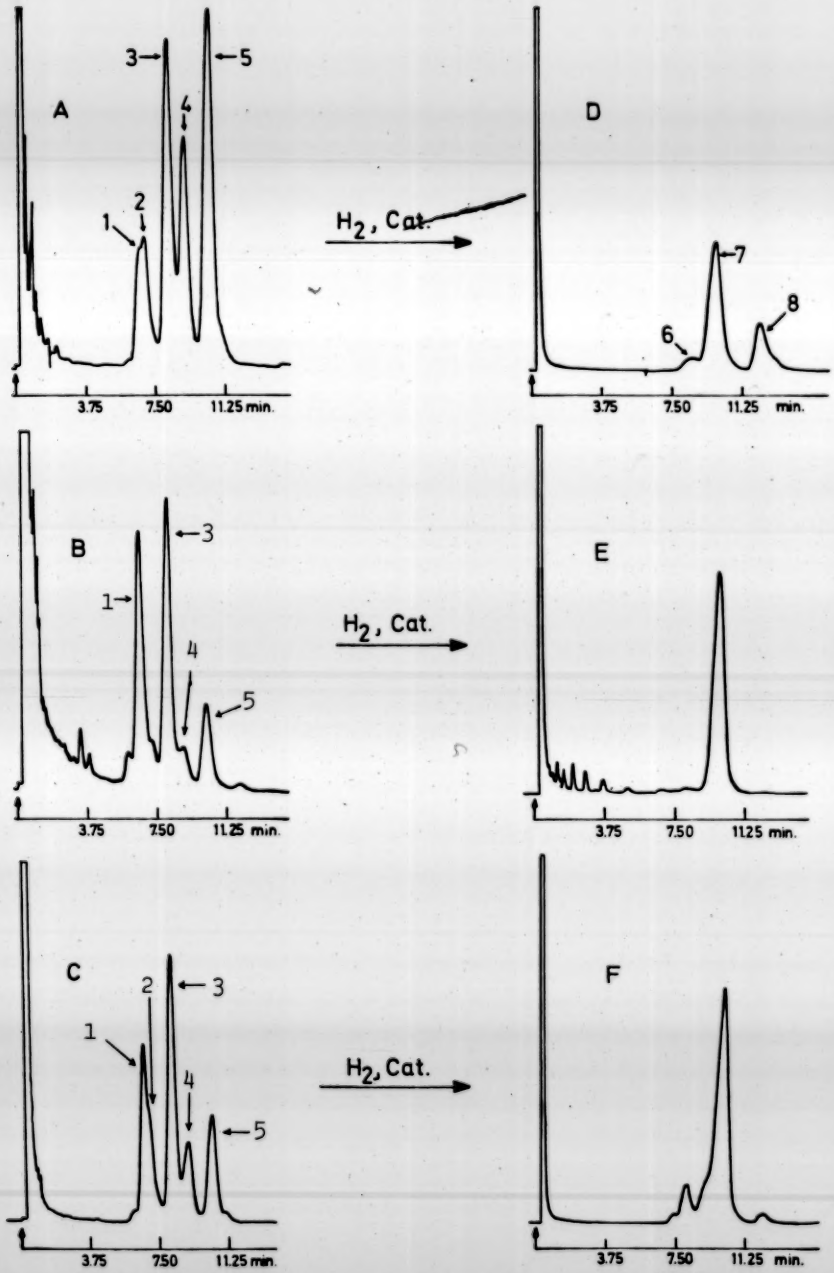


Fig. 36

the double bond.[23b,52] Treatment of pyrrolidine with n-butyllithium and 0.5 equivalents of cuprous iodide followed by addition of 51 (Fig. 36, path A), afforded 70% of a complex mixture consisting of Z,Z-; Z,E; and E,E- isomers; as well as the presumably (1-3) and (3-3) coupled isomers (Fig. 37A). The difference between this isomeric mixture, and those from the literature, prepared by the same procedure,[52] is the presence of Z,Z- and Z,E- geometrical isomers. These arise as a consequence of a different pattern of substitution in the starting allylic bromide 51. Assignment of the structure to these isomers was postponed until more information was available for correlation studies. In the second approach (Fig. 36, path B), 2-methylfarnesyl bromide (2 Z:2 E, 69:31) was treated with about 4.5 equivalents of nickel tetracarbonyl[53] in DMF for 3 hr at 25°, then 20 hr at 50°. The mixture obtained in about 24% yield consisted


A
1
2
3
4
5
3.75
7.50
11.25 min.
H2, Cat.
D
6
7
8
3.75
7.50
11.25 min.
B
1
3
4
5
3.75
7.50
11.25 min.
H2, Cat.
E
3.75
7.50
11.25 min.
C
1
2
3
4
5
3.75
7.50
11.25 min.
H2, Cat.
F
3.75
7.50
11.25 min.

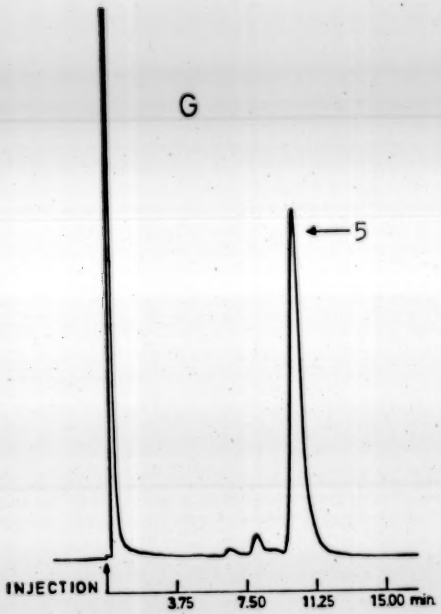


Figure 37. GC analyses of the reaction mixtures obtained as follows: coupling of 2-methylfarnesyl bromide (2Z:2E,2:98) with cuprous iodide-lithium pyrrolidide complex (A); reaction of 2-methylfarnesyl bromide (2Z:2E,69:31) with nickel tetracarbonyl (B); condensation of 2-methylfarnesyl bromide (2Z:2E,14:86) with nickel tetracarbonyl (C); hydrogenation of the first coupling reaction (D); hydrogenation of the second coupling reaction (E); hydrogenation of a purified fraction of the third coupling reaction (F); and of the substance separated by thiourea clathrate formation (G). Conditions: System A, 235° (chromatograms A,B,C, and G); System A, 215° (chromatograms D,E, and F).

of the same isomers found with the copper reagent, but in a different ratio (Fig. 37B). For the purpose of clarity, these isomers are numered as peaks (1), (2), (3), (4), and (5), in the order they emerge from the GC. Only these isomers will be considered for correlation studies. It is seen that the peak with the longest retention time in the second reaction (5), which presumably is the E,E-isomer, is about 14% of the mixture. In order to enhance the yield of this isomer, a second reaction was carried out starting with a different mixture of alcohol 36 (2Z:2E, 14:86). This time the reaction mixture, stirred for 17 hr at 42°, produced the isomeric mixture in 57% isolated yield, still containing only 16% of the E,E- isomer (Fig. 37C). Formation of the presumably regio-isomers was significantly increased in this reaction (peaks 2 and 4). The presence of regio-isomers was not detected in the previously reported nickel tetracarbonyl reactions.[54,55] Formation of these isomers in the present reaction carried out with the same coupling reagent can be rationalized by the argument presented for the formation of geometrical isomers in the cuprous iodide reaction. Thus, the reported allylic bromides[54,55] have a di- or trisubstituted C_2-C_3 double bond whereas in the present work this double bond is tetrasubstituted (as in 51).

Assignment of stereochemistry to these peaks is a result of the following studies. (a) Correlation of the gc relative retention times with those of the reactions dis-

cussed before suggest that peaks (1), (3),and (5) are the Z,Z-; Z,E; and E,E- isomers respectively.[54,55] This assigns peaks (2) and (4) to the (3-3) and (1-3) regio-isomers respectively, since (1-3) is expected to form in preference to the (3-3) isomer.[52] (b) Total hydrogenation of the cuprous iodide reaction mixture gave a mixture consisting of three major peaks by glc (Fig. 37D). It is seen that peak (7) amounts to 67% of the total, which is about the expected percent if peaks (1), (3), and (5) were geometrical isomers which became identical on hydrogenation. On the other hand, peaks (6) and (8) are about the same as peaks (2) and (4) in the starting unsaturated mixture. Analogous results are obtained upon hydrogenation of the nickel carbonyl reaction mixtures. The first of these reactions gave one major compound (Fig. 37E) with the same retention time as peak (7) described before; this compound was also obtained upon hydrogenation of the second nickel tetracarbonyl reaction (Fig. 37F), although a correlation of percentages can not be made with Fig. 37C. These correlation studies assume that peak (7) is only one and not several compounds with the same retention time. (c) Selective isolation of the isomer with the highest retention time (peak 5) by thiourea clathrate formation (Fig. 37G), a method that has proved effective in the separation of all-E squalene from its geometrical isomers.[56] (d) The nmr spectrum (No. 12) of the

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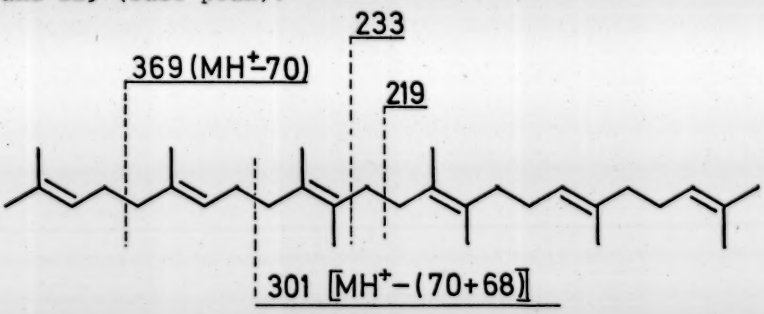
(56) (a) D. H. R. Barton, G. Mellows, D. A. Widdowson, and J. J. Wright, J. Chem. Soc., (c), 1142 (1971); (b) L. J. Goad and T. W. Goodwin, Biochem. J., 99, 735 (1966).

clathrate isolated isomer shows a singlet at 1.63 ppm for methyls trans to hydrogen at the 2, 6, 19, and 23 positions; the methyl cis to hydrogen (positions 2 and 23) appear at 1.67 as a singlet superimposed on a large singlet at 1.68 ppm due to methyls at the 10, 11, 14, and 15 positions.[51,57] On the other hand, the nmr spectrum (No. 13) of a fraction containing a larger concentration of peaks (1), (2), and (3) shows a different ratio of these methyl peaks, as expected for geometrical isomers. An aliphatic methyl peak is also observed at about 1 ppm, and vinyl protons at about 4.7 ppm, consistent with the presence of regio-isomers in the mixture. The chemical ionization-mass spectrum of the clathrate purified isomer shows a peak at m/e 439, corresponding to $C_{32}H_{54} + H^+$, and salient peaks at 369, 301, 233, and 219 (base peak).



(57) For the chemical shifts of methyl protons in terpenoid double bonds see: (a) R. B. Bates, R. H. Carnighan, R. O. Rakutis, and J. H. Schauble, Chem. Ind. (London), 1020 (1962); (b) R. B. Bates and D. M. Gale, J. Am. Chem. Soc., 82, 5749 (1960).

The studies discussed permit unambiguos assignment of peaks (1), (3), and (5) to the Z,Z; Z,E; and E,E- isomers, respectively.

b. Synthesis of 11-Methylsqualene (40), 10,15-Didesmethylsqualene (48), and 10-Desmethylsqualene (50).

These compounds were prepared by W.A. Vinson in these laboratories. The synthetic details are outlined in preliminary comunications of this work.[48]

c. Identification.

In order to obtain the hydrocarbon product from [1-^{3}H]-15 in sufficient quantity for identification, a large scale incubation was carried out (see experimental section). The incubation was terminated and worked up as usual. Glc analysis of an aliquot of the total mixture showed three major peaks (Fig. 38A); one with the same retention time as squalene (peak a), and two poorly resolved peaks at higher retention time (peaks b and c). Analysis by tlc-liquid scintillation counting showed approximately 30% of the radioactivity remained at the origin, while 70% was associated with a material closely resembling squalene (Rf 0.73, hexane). The polar spot at the origin is probably due to chemical enzymatic hydrolysis products.[13] The incubation mixture, therefore, was separated by preparative tlc and again analyzed by glc. The band at the origin corresponded to peak (c) (Fig. 38B), while the band with the same Rf as squalene showed peaks (a) and (b). Peak (c) was also observed in control incubations and was therefore

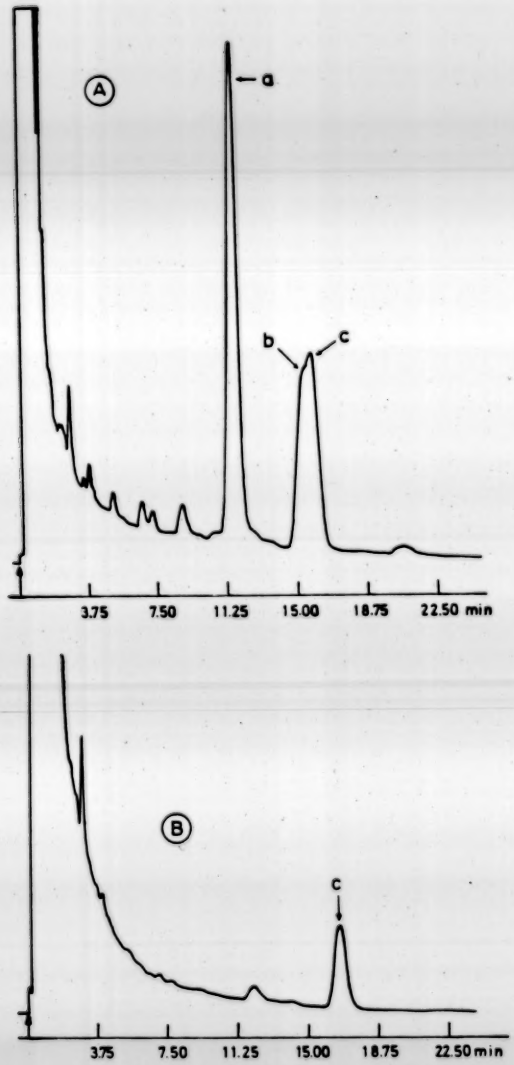


Figure 38. Gc analysis of the mixture obtained by a large scale incubation of 1-^{3}H -2-methylfarnesyl pyrophosphate (A); and of the polar component obtained by tlc separation of this incubation mixture (B). Gc conditions: System A, 220°.

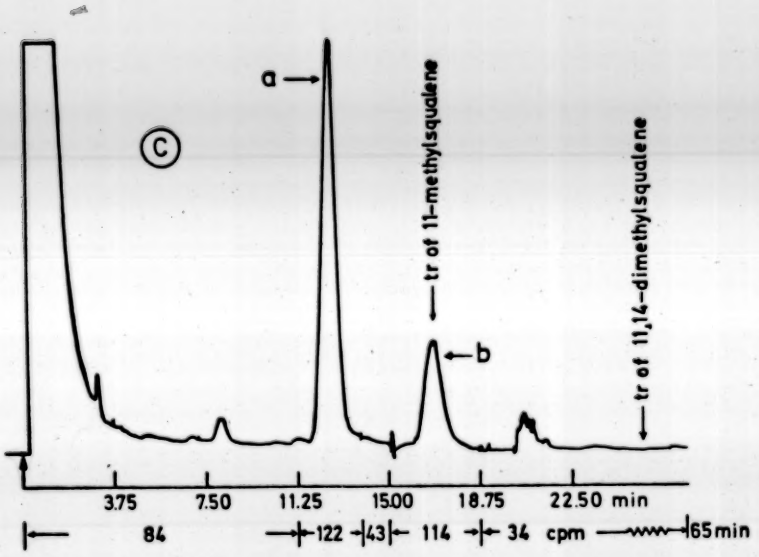


Figure 38C. GC analysis of the tlc purified labeled hydrocarbon obtained by a large scale incubation of [1-^{3}H]-2-methylfarnesyl pyrophosphate. GC conditions: System D, 200°.

discarded. Glc-liquid scintillation counting (Fig. 38C) of the hydrocarbon fraction showed that peaks (a) and (b) contained approximately equal amounts of radioactivity. Although peak (a) consists in part of endogenous squalene, another substance must be underneath, since the incubation did not contain any source of labeled squalene. However possible contamination with labeled farnesyl pyrophosphate could have arisen if commercial α-bromopropionate, the precursor of 2-methyl ester 37 (Fig. 13), were contaminated with α-bromoacetate. Glc analysis of the phosphono ester precursor 37 in Fig. 13 showed traces of a contaminant with the same retention time as triethyl phosphonoacetate. Nevertheless, glc analysis of alcohol precursor 36 did not reveal any detectable contamination by farnesol. Peak (b) coincided with authentic 11-methylsqualene, while 11,14-dimethyl squalene appeared at higher retention time, as indicated in Fig. 38C.

An aliquot of the hydrocarbon products, purified by tlc, was subjected to glc-mass spectrometry.[58] The first peak to emerge (a) had a molecular ion at m/e 410, corresponding to $C_{30}H_{50}$, and the fragmentation pattern characteristic of squalene.[49a] One of the observed fragmentation sequences begins with the elimination of 43 mass units (C_3H_7), and the other with elimination of 69 mass units (C_5H_9). The base peak appears at m/e 69, accompanied by a

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(58) This equipment is described in D. L. Gruenke, J. C. Craig, and D. M. Gier, Biomed. Mass. Spectrom., 1, 418 (1974).

peak at m/e 81 (Fig. 39A). It is evident that this spectrum corresponds to endogenous squalene, observed in all the incubations carried out in this work. The labeled product is apparently swamped out. The second peak (b) had a molecular ion at m/e 424 corresponding to $C_{31}H_{52}$ with a relative intensity of 1.9% of the base peak (m/e 69). Salient peaks were observed at m/e 355 (M-69) and 219[M-(69+68+68)], with relative abundances of 2.4 and 1.8% respectively (Fig. 39B). These peaks appear 14 mass units lower in squalene, while the peak at m/e 355 appears 14 mass units higher in 11,14-dimethylsqualene. The second radioactive peak was thus identified as 11-methylsqualene, formed by unsymmetrical condensation of 1 with [1-^{3}H]-15.

Formation of 11-methylsqualene implies that a normal substrate molecule was replaced during the first or second catalytic step.[13] These two alternatives are distinguishable, since a proton is exchanged only in the first, and not in the second, molecule that binds to the enzyme.[1,13] In order to establish whether 15 binds to the enzyme during the first or second catalytic step, two series of experiments were carried out in conjunction with W. A. Vinson and Dr. J. S. Wei. The first series was directed at measurement of tritium release into the medium during the incubation. The procedure is similar to that employed by H. C. Rilling for evaluating tritium release during incubation of [1-^{3}H]-1. In the reported procedure, the incubation is diluted with a small volume of methanol and the

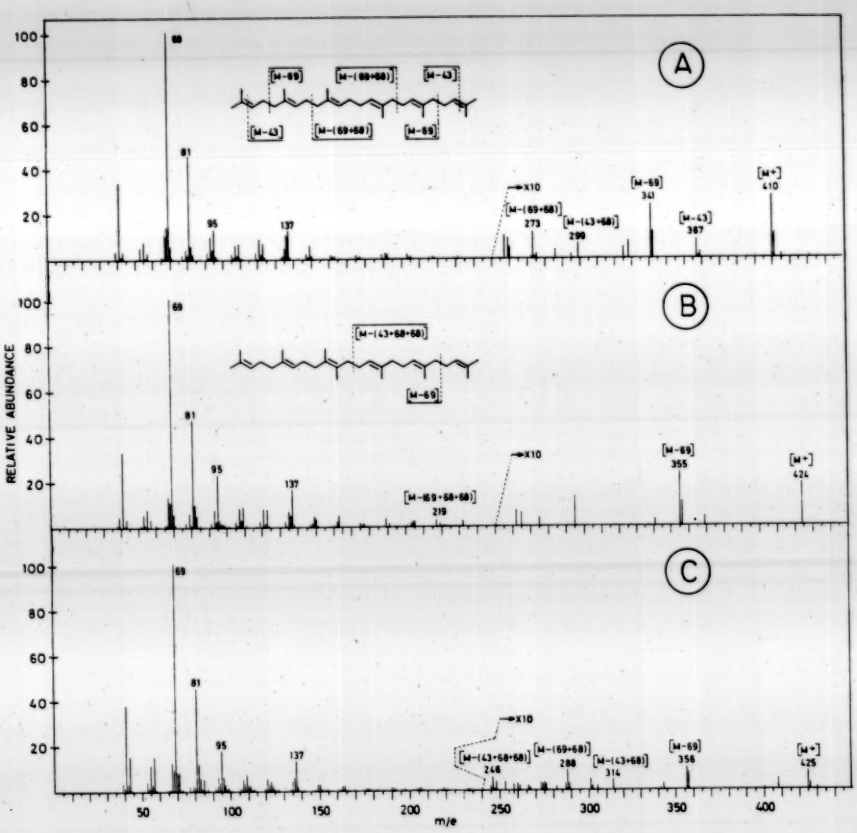


Figure 39. Mass spectra of endogenous squalene (A); of the compound obtained by incubation of 2-methylfarnesyl pyrophosphate (B); and of the substance obtained by incubation of 2-methylfarnesyl pyrophosphate and 1-dideuterio-farnesyl pyrophosphate (C).

resulting mixture partially distilled. An aliquot of the distillates is then subjected to liquid scintillation counting.[59] In the present work, analog [1-^{3}H]-15 was incubated as usual with an equimolecular amount of unlabeled 1. The incubation was terminated by addition of ethanol, the hydrocarbon products extracted with petroleum ether, and the extracts assayed by liquid scintillation counting. Part of the ethanol in the residual mixture was distilled and an aliquot of the distillates assayed by liquid scintillation counting. Control incubations were done at the same time with identical mixtures, except that the enzyme was inactivated by ethanol. The results of these experiments are summarized below.

Source	Radioactivity (dpm)
Distillate from incubation of [1-^{3}H]-15	176
Distillate from control incubation	125
Extracted hydrocarbon products	428

Incorporation of 15 only as a first substrate was expected to give an equal number of dpm as in the hydrocarbon product, since the alcohol precursor of 15 was nonstereospecifically labeled with tritium during reduction of the ethyl ester. The results obtained, however, do not permit clear differentialtion of 15 as a cosubstrate during the second catalytic step, since the radioactivity detected is within the sensitivity limits of the method. In the

(59) H. C. Rilling, J. Lipid Res., 11, 480 (1970).

second series of experiments, equimolecular amounts of 1-dideutero 1 and unlabeled 15 were incubated and the purified hydrocarbon obtained was subjected to glc-mass spectrometry.[58] The spectrum showed a molecular ion at m/e 425 and the base peak at m/e 69. Two salient peaks, retaining the central carbons, appeared at m/e 356(M-69) and 314[M-(43+68)]. The molecular ion peak, and that at m/e 356, are shifted upward by one mass unit relative to the corresponding peaks in the spectrum of unlabeled 11-methylsqualene (Fig. 39C). Analog 15, therefore, is utilized only as a substrate during the second catalytic step.

The steps leading to the identification of the hydrocarbon product obtained from incubation of [1-^{3}H]-16, were similar to those described for the identification of 11-methylsqualene. These studies were again done in conjunction with Dr. J. S. Wei and W. A. Vinson. Large scale incubation of [1-^{3}H]-16 gave a hydrocarbon product that was purified on column chromatography using neutral alumina and hexane-ethyl ether as solvent. Tlc analysis showed only one spot coincident with squalene. An aliquot was injected into a preparative glc and the fractions eluted were assayed by liquid scintillation counting (Fig. 40). A fraction (no peak detected) with the same retention time as authentic 10-desmethylsqualene (peak b) was associated essentially with all of the radioactivity. The major compound detected by the flame ionization detector was

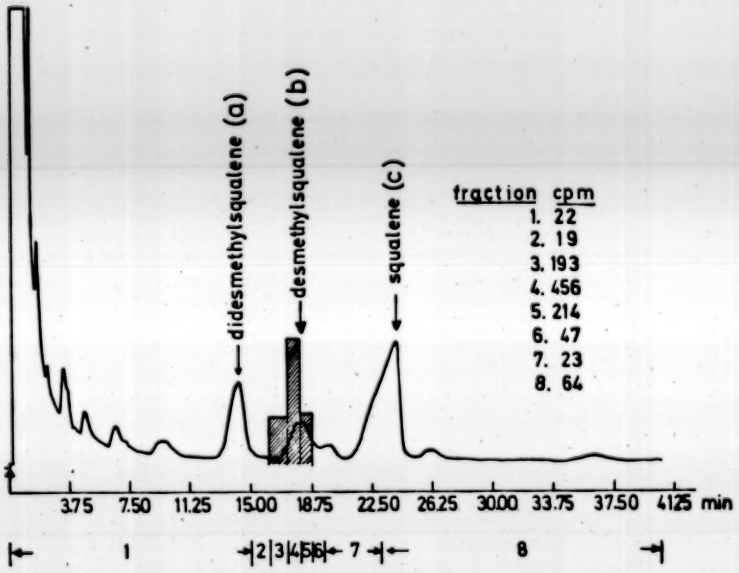


Figure 40. GC analysis of the mixture obtained by a large scale incubation of [1-^{3}H]-3-desmethylfarnesyl pyrophosphate. GC conditions: System D, 190°.

endogenous squalene, which appeared at a higher retention time (peak c) than the radioactive peak, while an authentic sample of 10,14-didesmethylsqualene has a lower retention time. The identity of the radioactive compound as 10-desmethylsqualene was firmly established by glc-mass spectrometry.[58] The mass spectrum showed a molecular ion at m/e 396 which corresponds to the molecular formula $C_{29}H_{48}$ (one methyl less than squalene). The base peak at m/e 69 was accompanied by a peak at m/e 81 (Fig. 41 A). These two peaks are characteristic of isoprenoid compounds, and were observed in all the mass spectra discussed here. Salient peaks retaining the central carbons were observed at m/e: 327(M-69), 285[M-(43+68)], and 259[M-(69+68)]. The corresponding peaks appeared in the spectrum of squalene (Fig. 39 A) 14 mass units higher. Further proof of this compound as 10-desmethylsqualene was obtained by comparing this spectrum with that of authentic material (Fig. 41B). Both spectra showed identical fragmentation patterns.

Studies to determine by tritium release whether [1-^{3}H]-16 binds to the enzyme during the first or second catalytic step were also carried out as described previously. The results of these studies are given below.

Source	Radioactivity (cpm)
Distillate from incubation of [1-^{3}H]-16	6500
Distillate from control incubation	6500
Extracted hydrocarbon	115000

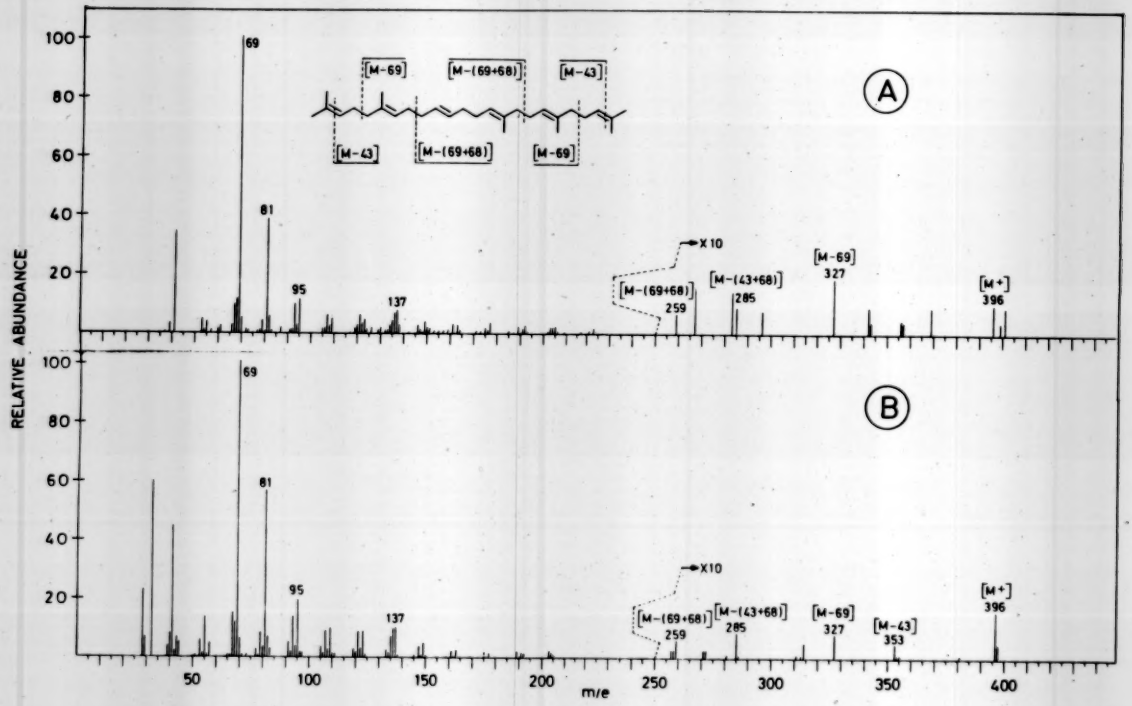


Figure 41. Mass spectra of the substance obtained by incubation of 3-desmethylfarnesyl pyrophosphate (A); and of an authentic sample of 10-desmethylsqualene (B).

As before, binding of [1-^{3}H]- 16 to the enzyme during the first catalytic step was expected to release as much tritium into the medium as was found in the extracted hydrocarbon, since the alcohol precursor (31) was racemically labeled during reduction of aldehyde 46. Interference by an isotope effect during enzymatic proton removal was ruled out, since it has been shown that the process is stereospecific, without an internal isotope effect.[3c] Although no tritium release was observed, the sensitivity of the assay did not permit detection of less than 230 cpm; this corresponds to 0.2% of the incorporated radioactivity which is therefore the maximun amount possible of hydrocarbon derived from [1-^{3}H]-16 as first substrate. Analog 16 thus binds to the enzyme during the second catalytic step.

Tritium labeled analogs 17, 20, and 22, which were not incorporated into hydrocarbon products, might have been incorporated only into nonextractable presqualene-like compounds unable to complete the sequence. Therefore, tritium release experiments were also carried out for these compounds in a similar manner as those described above.

The results of these experiments are tabulated below.

Source	Radioactivity (dpm)
Distillate from incubation of 17	31
Distillate from control incubation	29
Distillate from incubation of 20	30
Distillate from control incubation	29
Distillate from incubation of 22	233
Distillate from control incubation	254

The results obtained rule out these analogs as first substrates. The fact that no enzymatic tritium release was observed does not rule out the incorporation of these analogs into non-hydrocarbon products as second substrates, however.

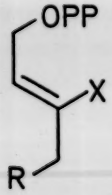
Part A and B Discussion and Conclusions

The work discussed in the first part of this chapter established that analogs 15-25 of farnesyl pyrophosphate are inhibitors of squalene synthetase, binding to the enzyme in competition with the substrate. The results of these studies indicate that attachment to the active site is determined mainly by pyrophosphate bonding forces, assisted by relatively non-specific lipophilic interactions. The importance of the pyrophosphate moiety is emphasized by the great difference in the inhibitory powers of the free alcohols, mono-, and pyrophosphates. The strong requirement for a pyrophosphate moiety suggests a pyrophosphate binding area at the active site of the enzyme. The nature of the alkyl group seems to be less critical, since appreciable inhibition was observed despite drastic modifications of the hydrocarbon structure.

Analogs which retain the C_{15} triply-unsaturated chain are in general more potent inhibitors than saturated analogs. For example, analog 15 produced twice as much inhibition as saturated analog 25, although both have the same apparent length of hydrocarbon chain. The length of the hydrocarbon chain appears to be important for binding, since saturated analog 23 is slightly more active than the analogous shorter analog 22. On the other hand, shortening of the hydrocarbon chain by a dimethylallyl unit, as in 21, considerably decreases binding, as demonstrated by the poor inhibition observed (see Fig. 18).

The differences in binding can reasonably be explained by the argument that changes in the stereochemistry and length of the molecule prevent proper fit into the active site. A second possible explanation is that the double bonds are involved in binding through π interactions with specific areas of the active site. Loss of these interactions upon hydrogenation would result in decreased inhibition. Although binding is in part due to the pyrophosphate moiety, a lipophilic binding area must also be present, since the different degrees of inhibition are a consequence of variations in the hydrocarbon chain. For example, it can be argued that analog 23, being a few atoms longer than 22, has more points of attachment to such a lipophilic region. This implies tighter binding to the active site, and consequently, a slight increase in inhibition.

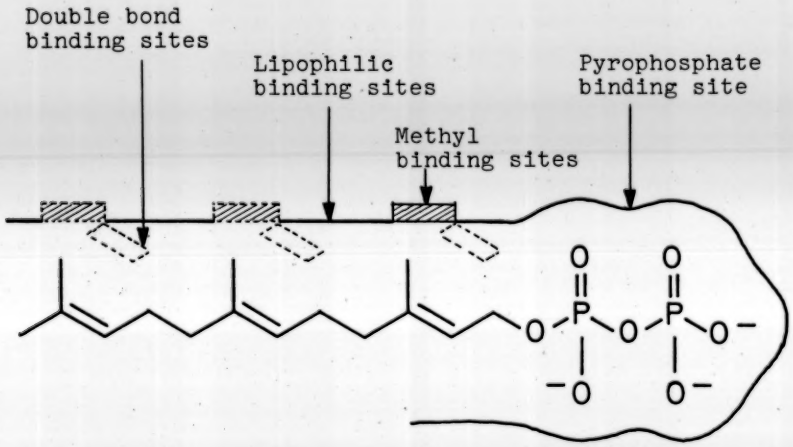
Although substitution at C-2, C-3, and C-4 is well tolerated, a marked difference in activity is observed. Analogs 16, 17, and 18 have the same molecular features, except for the substituent at the 3-position, which therefore determines the differences in observed inhibition (17>16>18).



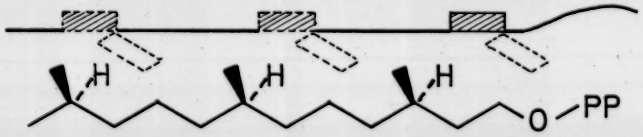
	X	Relative Inhibition (%)
16	H	72
17	CH_3CH_2	82
18	I	64

An attractive explanation for these differences in binding is based on the electronic properties of the substituents. An ethyl group is electron releasing in comparison with H and I. The electronic density between C_2-C_3 in 17 is therefore richer than in 16 or 18, and 17 would thus be expected to bind more tightly through π interactions at the receptor site. The same reasoning can be applied to analogs 15, 19, and 20. Analog 15, with methyl groups at C-2 and C-3, is slightly higher in potency than 17, in agreement with the higher electron density due to increased alkyl substitution. Analog 19, with a C-2 iodo instead of methyl group, has the same activity as 18 as predicted by this hypothesis, since the same inductive effect is present in 18 and 19. Analog 20, in which the electron withdrawing inductive effect of the thiomethyl group is less pronounced, since the substituent is at the C-4 position, is slightly more active than the iodo analogs. If steric effects alone are considered, the fact that 3-ethyl analog 17 is more active than 3-desmethyl analog 16, cannot be accounted for. However, if the enzyme has a hydrophobic region coincident with the C-3 area of the analog molecule, an ethyl group would be better bound than hydrogen. The iodo and thiomethyl substituents may also reduce binding due to steric interactions with certain areas of the active site.

Based on the observations so far discussed, a crude picture of a possible active site can be drawn.



The decrease in binding upon hydrogenation of the double bonds can be visualized by comparing saturated analog 25 with this hypothetical picture. There are no double bond interactions and the methyl substituents are not in proper positions for maximum binding. Comparing this type



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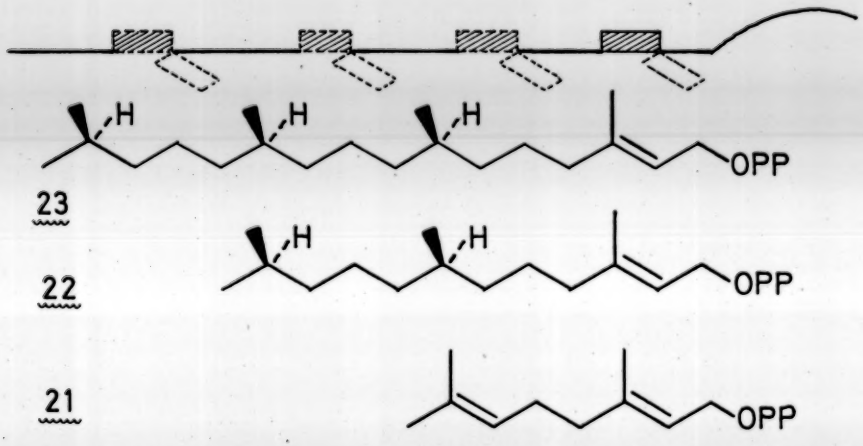
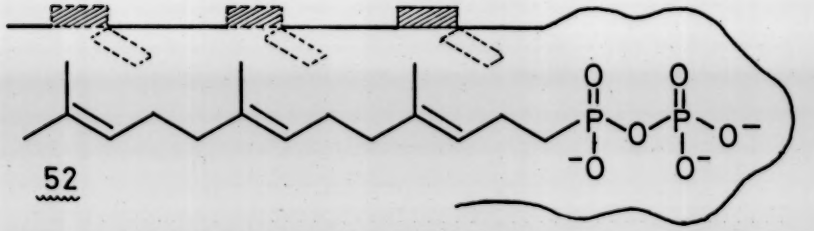
of drawing for analog 22 and 23 rationalizes the slight increase in inhibition by 23 over 22, since 23 has more lipophilic area in contact with the enzyme than 22. In the case of 21, which is less lipophilic, there is less binding, and consequently less inhibition.



Recent studies have shown that farnesylmethylphosphonophosphate 52 inhibits the biosynthesis of squalene from mevalonate.[60] It may be that this inhibition is caused by competition between 52 and 1 for the active site, since 52 has all the features required for strong binding to the enzyme, as seen in the representation below.



(60) E. J. Corey and R. P. Volante, J. Am. Chem. Soc., 98, 1291 (1976).

The results of the studies with artificial substrates indicate rather strict catalytic requirements for squalene synthetase. Incubation of analogs 15, 16, 17, 20, and 27, which have varied chemical structures, only led to acceptance of 2-methyl analog 15 and 3-desmethyl analog 16 as cosubstrates. Exclusive incorporation of these analogs during the second catalytic step is confirmed by the lack of tritium release from 15 and 16, since it has been established that in the normal reaction only the first molecule of substrate that binds to the enzyme (first catalytic step) undergoes proton exchange (Fig. 6).[13] Furthermore, these analogs bind to the active site of the enzyme, as confirmed by the competitive nature of the inhibition observed. However, they fail to form enzyme-substrate covalent complexes (E-I). This conclusion derives

$$E + IPP \rightleftharpoons E\text{-}IPP \not\rightarrow E\text{-}I + PP$$

from the following observations (1) the fact that these analogs are cosubstrates during the second catalytic step implies that the normal substrate undergoes the first step, e.g., formation of an enzyme-substrate covalent complex (E-F); (2) the possibility of forming such a complex at one catalytic site without further progress to products

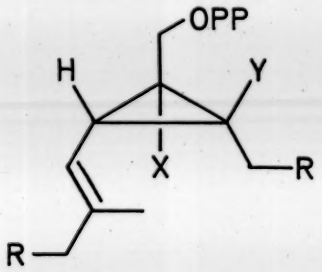
$$E + FPP \rightleftharpoons E\text{-}FPP \longrightarrow E\text{-}F + PP$$

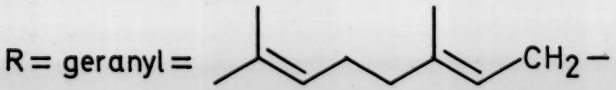
(no tritium release) is ruled out, since we found that the enzyme is not irreversibly inhibited by the analogs. These observations suggest that the enzyme contains two catalytic sites, one where the normal substrate undergoes complex

formation, and a second where the analogs can be incorporated. These could be different topographical regions of the enzyme, or a single catalytic region which undergoes a conformational change on reaction with the first substrate.

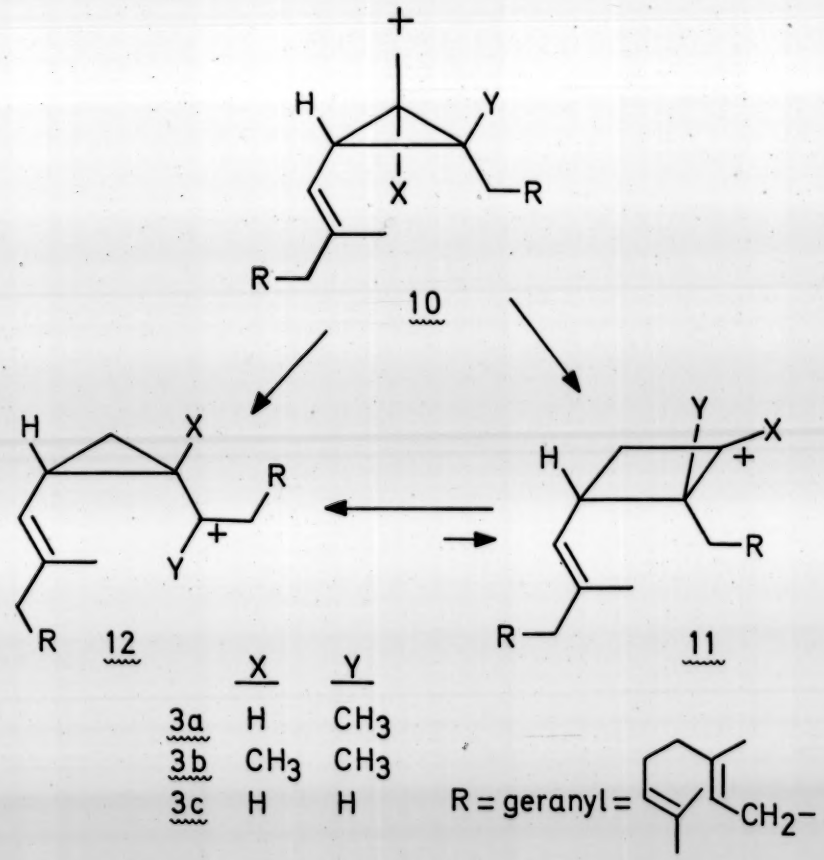
The observations discussed suggest that formation of 11-methylsqualene (49), and 10-desmethylsqualene (50) proceed through intermediates 3b and 3c, respectively, in analogy with the normal reaction through 3a (Figs. 6, 7, and 8).

	X	Y
3a	H	CH_3
3b	CH_3	CH_3
3c	H	H





In the introduction evidence was cited that the main energy barrier in the formation of compounds with the squalene skeleton from cyclopropyl carbonium ions is the rearrangement of 10a to give 11a (p.13). Opening of the cyclopropyl carbinyl cation 10b to give cyclobutyl cation 11b



should be favored over the analogous reaction of 10a to form 11a, since the extra methyl group inductively stabilizes the cation formed. Solvolysis studies of methyl sub-

stituted cyclopropylcarbinyl analogs[61] suggest that this rate enhancement might be in the order of five fold.

If indeed the enzymatic rearrangements follow the model studies,[17] further reaction of 11a may be diverted by steric and electronic perturbations introduced by the methyl group.[62] It may be that the still unidentified radioactive compound obtained from incubation of [1-^{3}H]-15, with the same retention time as squalene (peak a in Fig. 38 C), derives from a side reaction of 11b. Rearrangement of 10c to 12c on the other hand, should not be favored compared to the analogous rearrangement of 10a to 12a, since methyl stabilization present in 12a is lost in 12c.[62] Estimates of the decrease in rate of this conversion may again be in order of five fold.[61] Nevertheless, comparing the stabilities of these cationic species with that of model methyl analogs,[62] the equilibrium should still lie towards 12, as found for 12a.[17] The differences in reactivity are not so large that enzymatic catalysis cannot overcome them.

It has been shown that analogs 15 and 16 bind to the active site of the enzyme in competition with the normal substrate. However, it is uncertain whether the inhibition observed is caused by non-productive binding of these

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(61) P. v. R. Schleyer and G. W. Van Dine, J. Am. Chem. Soc., 88, 2321 (1966).

(62) Two reviews concerning with the reactivity of these species are (a) H. G. Richey, Jr., in Carbonium Ions, Vol. III, G. O. Olah and P. v. R. Schleyer, Eds., Wiley-Interscience, New York, 1972, chapter 25. (b) K. B. Wiberg, B. Andes Hess, Jr., and A. J. Ashe, III., Ibid., chapter 26.

analogs to (a) the first catalytic site, to form a Michaelis complex (E-IPP), or (b) exclusive binding to the second catalytic site, where they are poorer cosubstrates than 1. Therefore it cannot be determined if the inability of these analogs to act as first substrates in the synthesis of 49 or 50 is because they bind to the first catalytic site and fail to meet catalytic requirements or because they do not bind to this catalytic site.

The fact that 3-desmethyl analog 16 is not incorporated as a first substrate, and that the enzyme is not irreversibly inhibited by this analog, demonstrates that the enzyme does not recognize a substrate without the C-3 methyl group during the first catalytic step. These observations indicate that the C-3 methyl group is essential for enzymatic formation of squalene. Thus, formation of an enzyme-substrate covalent complex requires a methyl group at the C-3 position. Analog 15 has methyl groups at C-2 and C-3, but it also does not form such a complex, indicating a sterically congested first catalytic site. This observation is reflected by the 4-thiomethyl analog 20, which was not incorporated at all, although electronic effects may be involved in the case of this analog. The C-3 methyl group could be involved in immobilizing the substrate by binding to a specific methyl binding site, in inducing the catalytic conformation of the enzyme, or in a combination of both. It is clear, in any case, that the C-3 methyl group is essential prior to or during the first catalytic step. Failure of

3-ethyl analog 17 to act as first substrate demonstrates steric constraints compatible with methyl binding at the first catalytic site. However, the fact that no hydrocarbon formation or tritium release was observed does not exclude the possibility of 17 undergoing reaction during the second catalytic step to give only presqualene-like products, since these would not have been detected. Nonetheless, the fact that 3-desmethyl analog 16 is a cosubstrate, even if a poor one, in the second catalytic step, indicates that methyl binding is not as essential here as is in the first catalytic site. Tetrahydro analog 22 retains the C-3 methyl group and C-2 double bond, which are needed for condensation at either catalytic site (Fig. 6). Nevertheless, this analog is not incorporated into products. An explanation for this lack of incorporation is that on hydrogenation of the double bonds the specific binding that hypothetically triggers catalysis is lost. A possible implication is that the C-7 and C-11 methyl groups may also be essential for catalysis during one or both of the catalytic steps.

From the results of these studies a "hypothetical" active site can be drawn. Normal reaction will follow the steps outlined in Figs. 42 and 43. Binding of the normal substrate, mediated by the pyrophosphate group, could be enhanced by the methyl groups, double bonds, hydrocarbon chain, and the essential magnesium ion (Fig. 42 A).[13] These specific interactions, particularly methyl binding, could induce the catalytic conformation of the enzyme, exposing a nucleophilic group in the enzyme. This would account for

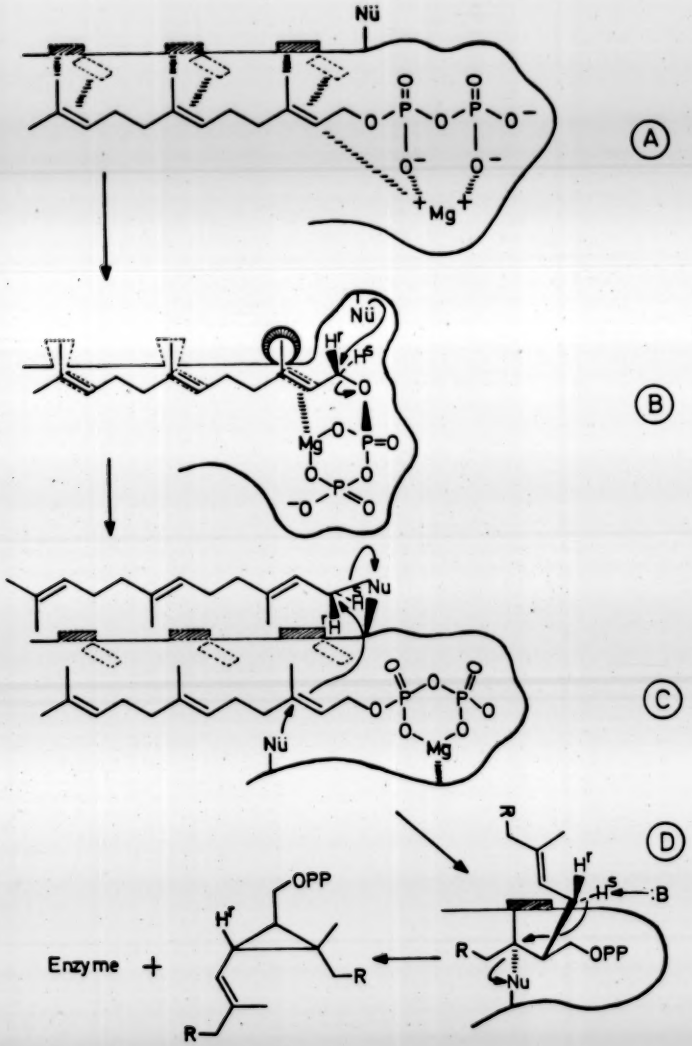

Figure 42. Hypothetical active site of squalene synthetase in which one catalytic site is considered for the formation of presqualene pyrophosphate.

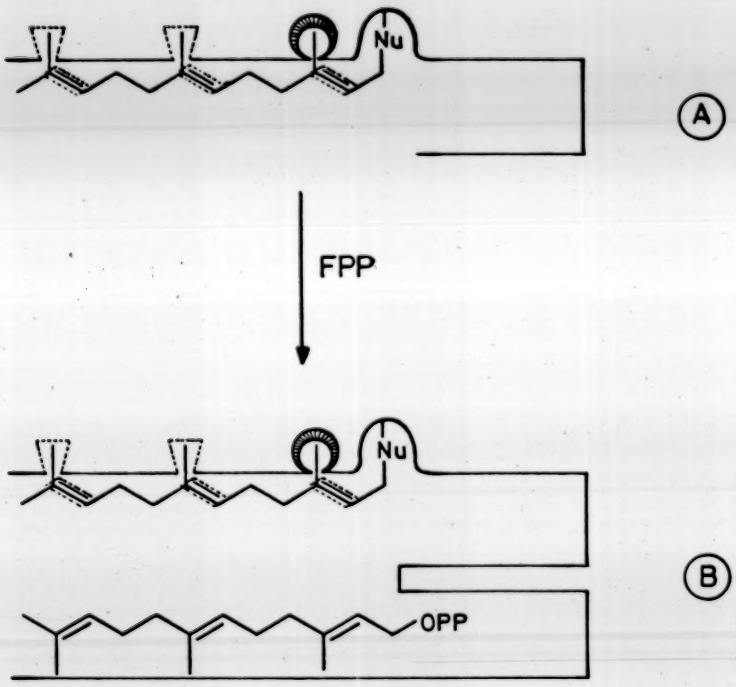

Figure 43. Hypothetical active site of squalene synthetase in which two catalytic regions are involved in the coupling of two molecules of farnesyl pyrophosphate.

the requirement of the methyl group and the displacement of the pyrophosphate moiety (Fig. 42B). Although there is no evidence for specific binding sites for the other methyl groups, such sites could account for the lack of incorporation of the tetrahydro analog 22, the reduced inhibition produced by the saturated analogs and geranyl pyrophosphate, and the conversion of geranylgeranyl pyrophosphate to lycopersene.[49c] The need for magnesium ion can be visualized as participation with the pyrophosphate group to form a cyclic complex interacting with the C-2 double bond in an analogous manner to that postulated for the participation of this ion with adenosine triphosphate in the activation of fatty acids.[63] Displacement of the pyrophosphate group would invert the configuration at C-1 forming a farnesyl-enzyme covalent complex.[13] Further reaction would then require a second catalytic site. Assuming that the first catalytic site undergoes a change in conformation, the farnesyl chain could be displaced from the lipophilic binding site and a second substrate molecule attached. Binding of the second molecule of substrate might induce a different conformation of the enzyme, again exposing a nucleophilic group that upon interaction with the C-2 double bond of the second substrate molecule displaces the first nucleophilic group of the enzyme, inverting the configuration at C-1 of the first molecule of substrate to the original one (Fig. 42 C).[1,13] These

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(63) J. W. Cornforth, J. Lipid. Res., 1, 3 (1959).

changes in stereochemistry could place H^S close to a basic group which, upon removal, would give presqualene pyrophosphate (Fig. 42 D).

An alternative for incorporation of the second substrate molecule is to consider a second catalytic site, located in a different topographical area of the enzyme (Fig. 43 A,B). The mechanism would be the same as before, except that the two distinct sites are involved.

The results of our studies suggest that of all the analogs studied, except possibly 3-iodo analog 18, none may be incorporated as first substrate. Analogs 15, 16, 17, 20 and 22 have already been described. For the same reasons by which analog 22 is not a substrate, saturated analogs 23 and 25 are not expected to be substrates. Propargylic analog 24 would not be expected to form complexes 26 or 27, since it does not have the required C-3 methyl group. Analog 18, on the other hand, has the three double bonds and two of the three vinyl methyls of the farnesyl chain, while the first one is replaced by an iodo group. It is questionable, however, if this analog can act as first substrate, since the substituent introduces strong steric and electronic perturbations to the molecule at the C-3 carbon, a highly specific center.

Failure to observe irreversible inhibition with these analogs can be rationalized in part, considering that formation of an irreversible complex most easily occurs if the analog is catalytically accepted as a first substrate. If

the analog is only accepted in the second catalytic step, it would have to differentially meet the catalytic demands of the enzyme in order to form a locked enzyme-substrate-inhibitor covalent complex.

PART C

PRENYL SUBSTITUTED CYCLOBUTANONES. SYNTHESIS AND PRELIMINARY STUDY AS SQUALENE SYNTHETASE INHIBITORS

1. Introduction

The function of an enzyme, like that of any other catalyst, is to make the transition state for a reaction easier to reach. One can rationalize at least a part of their catalytic action if enzymes possess an unusual affinity for the altered substrate in the transition state, exceeding that for the substrate itself. Recent studies on enzymatic catalysis suggest that binding in the transition state is very tight indeed.[64] Compounds which resemble the transition state structure of the substrate would therefore bind to the enzyme more tightly than the normal substrate, inhibiting conversion of the latter to products.

Model studies for the conversion of presqualene pyrophosphate 3 to squalene 2 (p.13) suggest that the highest energy barrier in the rearrangement sequence lies near cyclobutyl cation 11,[17a] the pyrophosphate of which has been mentioned as a possible discrete intermediate.[9e] If model studies do, in fact, reflect the enzymatic mechanism, the transition state for the enzymatic rearrangement sequence should also approximate 11.[65] Cyclobutanone analogs of cat-

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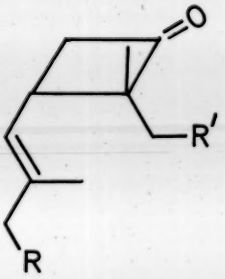
(64) (a) G. E. Lienhard, Science, 180, 149 (1973); (b) R. Wolfenden, Accts. Chem. Res., 5, 10 (1972); (c) R. N. Linquist, chapter 2 in "Drug Design", Vol. 5, E. J. Ariens, Ed., Acad. Press, N. Y. 1975.

(65) G. S. Hammond, J. Am. Chem. Soc., 77, 334 (1955).

ion 11, in which the charged atom is replaced by a similarly hybridized and polarized carbonyl carbon, might therefore function as "transition state" inhibitors of the enzyme squalene synthetase.[64] The cyclobutanones would also be useful as precursors to cyclobutanol derivatives for further mechanistic and model studies.

Our work on the synthesis of cyclobutanones 53 a-c is discussed here. Preliminary bioassays have been carried out on 53a, 53c, and pyrophosphate 54.



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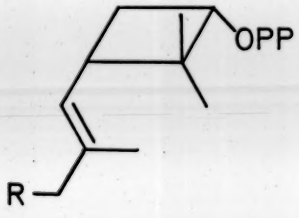
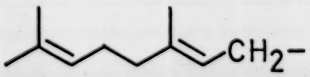


54

R = geranyl= 

R′= a) H b) geranyl c) tetrahydrogeranyl

2. Photochemical Reactions

The initial synthetic approach to the preparation of cyclobutanone 53b was based on the postulated photochemical reaction of farnesal (55a) with 1,1-dimethoxyethylene[66] (Fig. 44) to give aldehyde 56a. This aldehyde, upon treatment with phosphorane 57,[10c] followed by mild acid hydrolysis, was expected to give 53b. In a typical reaction, a solution of 55a and excess 1,1-dimethoxyethylene in hexane was irradiated in a quartz cell for 5 to 11 hr with a high-pressure mercury arc (Hanovia 450 W) using a Corex filter.[66]

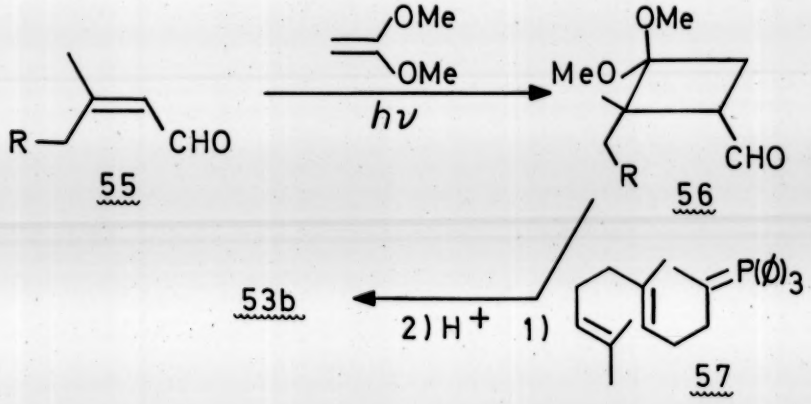


Fig. 44

Multiple product formation was always observed. Among the products detected were starting aldehyde 55a, an unconjugated aldehyde containing no methoxy groups, and probably the ex-

(66) E. J. Corey, J. D. Bass, R. LeMahieu, and R. B. Mitra, J. Am. Chem. Soc., 86, 5570 (1964).

pected compound (ir, nmr, CIMS) in low yields (<5%).

Model reactions carried out with citral(55b)and 1,1-dimethoxyethylene in hexane also led to multiple product formation. Changing the solvent to benzene and replacing 1,1-dimethoxyethylene with 1,1-dichloroethylene[67] led to polymerization of the latter. Since it is known that citral can undergo self-cyclization when irradiated by a medium-pressure arc (125 W),[68] while α,β-unsaturated aldehydes such as cinnamaldehyde and crotonaldehyde react photochemically with 2-methyl-2-butene to give oxetanes and cyclobutane aldehydes,[69] a different approach was initiated (Fig. 45).

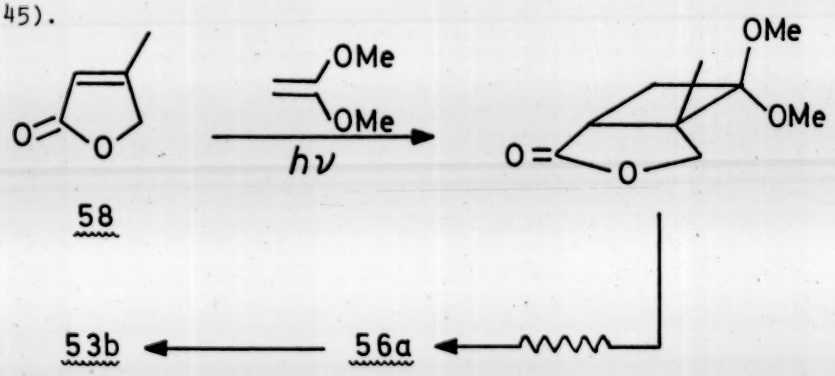


Fig.45

(67) P. Boyle, J. A. Edwards, and J. H. Fried, J. Org. Chem., 35, 2560 (1970).
(68) R. R. Cookson, J. Hudec, S. A. Knight, and B. R. D. Whitear, Tetrahedron, 19, 1995 (1963).
(69) (a) N. C. Yang, Pure Appl. Chem., 9, 591 (1964); (b) D. R. Arnold, Adv. in Photochem., 6, 301 (1968).

Lactone 58[70] and excess 1,1-dimethoxyethylene in hexane were irradiated as before for three days. The crude reaction mixture consisted primarily of the starting materials by nmr analysis. Only small quantities (<10%) of a compound with methoxy groups could be detected. Failure of these photochemical reactions led to a search for a more suitable procedure.

3. Synthesis of Cyclobutanones

The formation of cyclobutanones by polar cycloaddition of keteneimmonium cations with conjugated dienes appeared promising, since it was reported to proceed in high yields, with selectivity for the terminal double bond in 1,3-dienes, and with no Diels-Alder adduct formation.[71] The sequence outlined in Fig. 46 was therefore pursued.

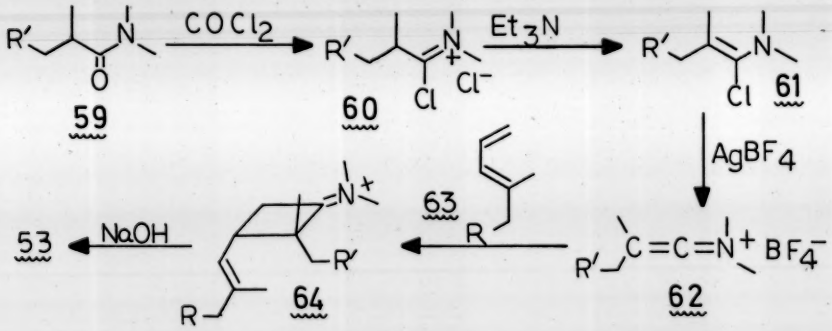


Fig. 46

(70) W. W. Epstein and A. C. Sonntag, J. Org. Chem., 32, 3390 (1967).

(71) J. Marchand-brynaert and L. Ghosez, J. Am. Chem. Soc., 94, 2870 (1972).

a. Synthesis of 4,8,12-Trimethyl-1,3(E),7(E),11-tridecatetraene (63)

Conjugated diene 63 was obtained in 94% yield by Wittig condensation[72] of E,E-farnesal with the ylide generated by treatment of methyltriphenylphosphonium bromide with n-butyllithium. When recently prepared, this compound shows one spot by tlc and one peak by glc; however, after prolonged exposure to air, a second spot appears at the origin on tlc, and several peaks with higher retention times than the diene on glc. Although the impurities, probably oxidation products, could be removed by slow bulb-to-bulb distillation, satisfactory elemental analysis could not be obtained. Nevertheless, the structure of this compound was established by spectroscopic analysis. In the infrared spectrum (No. 2), two weak bands characteristic of a terminal vinyl appeared at 3.25 and 3.58μ, while bands corresponding to a conjugated diene appeared at 6.1 and 6.20μ.[73] The nmr spectrum (No. 14) was easily rationalized and was consistent with a model from the literature.[74]

Vinyl protons
4.6-5.1 m,2H
5.5-5.8 m,1H
6.1-6.8 m,1H

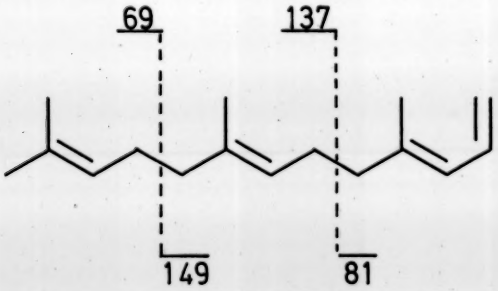
Vinyl protons
4.83-5.34 m,(2H+2H)
5.68-6.01 m,1H
6.28-6.93 m,1H

(72) H. Yamamoto, Ph. D. Thesis, Harvard University 1971, p. 112.

(73) K. Nakanishi, Infrared Absorption Spectroscopy, Holdenday, Inc., San Francisco, 1969.

(74) E. J. Corey and D. E. Cane, J. Org. Chem., 34, 3053 (1959).

All the signals not shown in the diagram appeared in the proper region with good integration. The chemical ionization-mass spectrum showed a molecular ion (MH^+) at m/e 219 corresponding to $C_{16}H_{26}$; salient fragmentation peaks characteristic of terpene hydrocarbons appeared at m/e 149 (M-69), 137[M-(69+68)], 69, and 81.



b. Synthesis of N,N-Dimethylisobutyramide (59a)

The title compound was obtained by bubbling anhydrous N,N-dimethylamine through a solution of isobutyryl chloride.[75]

c. Synthesis of N,N,2,6,10-Pentamethyl-5(E),9-undecadien-1-oic Amide (59b)

The synthesis of dimethylamide 59b is outlined in Fig. 47. Reduction of geranylacetone[29] with sodium borohy-

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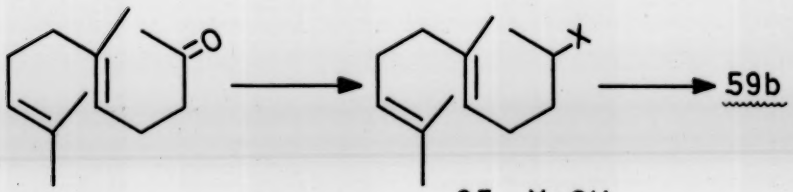
(75) (a) Y. Miron and H. Morawetz, Macromolecules, 2, 162, (1969); (b) H. Rapoport and R. M. Bonner, J. Am. Chem. Soc., 72, 2783 (1950).



65, X=OH
66, X=OTs
67, X=CN
68, X=COOH
69, X=CON

Fig.47

dride afforded alcohol 65 in quantitative yield. Treatment of this alcohol with p-toluenesulfonyl chloride gave tosylate 66 in good yield. Displacement of the tosylate group by cyanide ion in dimethylsulfoxide led to poor yields of 67. The side products of this reaction behaved like geranylacetone, alcohol 65, and hydrocarbons, as judged by tlc and glc. These, however, could be reduced considerably by carrying out the reaction in hexamethylphosphoramide. Basic hydrolysis of nitrile 67 in ethyleneglycol[76] gave the salt of 68, whose contaminants could be removed by extraction with dichloromethane. Acidification gave 68[77] in 72% overall yield. Activation of 68 as an acid chloride was attempted. Treatment of 68 with thionyl chloride at 70°, or at room temperature, gave a dark mixture which was treated with anhydrous

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(76) M. S. Newman and R. M. Wise, J. Am. Chem. Soc., 78, 452 (1953).

(77) K. Mori and M. Matsui, Tetrahedron, 26, 2801 (1970).

dimethylamine. In both cases, before or after amine treatment, the same multiple spots were observed on tlc, none of which corresponded to amide 59b. A different approach based on the reactivity and mildness of N,N-carbonyldiimidazole[78] was therefore undertaken. Imidazolide 69 was obtained by treatment of acid 68 with N,N-carbonyldiimidazole (Fig. 48). The reagent was in turn generated by reaction of imidazole with phosgene.[78b] Treatment of 69, without isolation, with N,N-dimethylamine finally gave 59b in 92% yield (ir. No. 3).

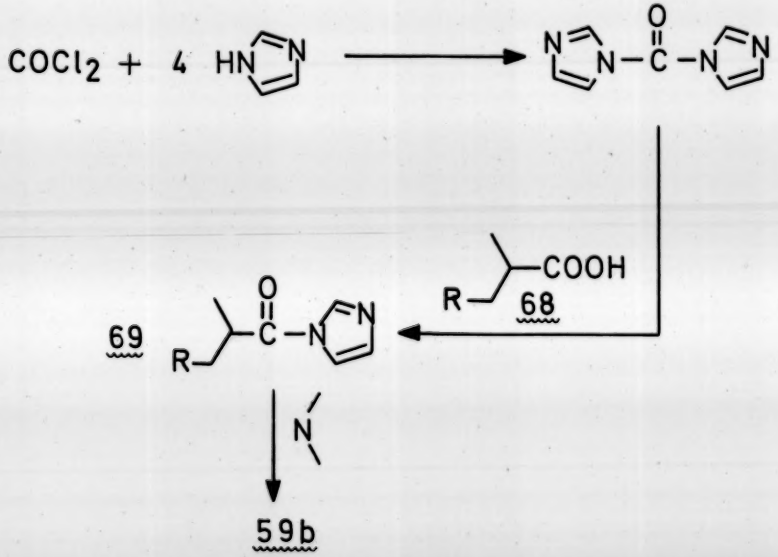


Fig. 48

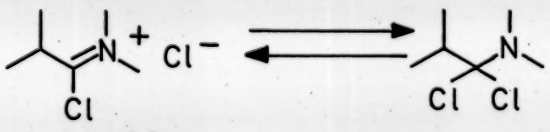
(78) (a) For review see H. A. Staab, Angew. Chem., Internat. Ed., 1, 351 (1962); (b) H. A. Staab and K. Wendel, Org. Syn., 48, 44 (1968).

d. Synthesis of N,N,2,6,10-Pentamethylundecan-1-oic amide (59c)

Catalytic hydrogenation of 59b, using 10% palladium-on-charcoal, gave saturated amide 59c in 97% yield.

e. Synthesis of 3-(2,6,10-Trimethylundeca-1(E),5(E),9-trienyl)-2,2-dimethylcyclobutanone (53a)

Reaction of N,N-dimethylisobutyramide (59a) with a commercial 12% solution of phosgene in benzene, or with phosgene gas, gave nice white crystals in more than 60% yield. The nmr spectrum (No. 15) was consistent with the expected chloroimminium chloride 60a.[79] However when anhydrous triethylamine was injected into the nmr tube, the signals reverted instantly to those of the starting amide.



60a

δ		
1.35	s,	6H
3.27	m,	1H
3.33	s,	6H

The probable explanation for this result appeared to be that triethylamine was contaminated with water, since compounds like 60a are known to react with water very easily.[79b,80] The amine was therefore rigorously dried by fractional distillation from calcium hydride, refluxing the distillate

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(79) (a) H. Babab and A. G. Zeiler, Chem Rev., 73, 75 (1973); (b) L. Ghosez, B. Haveaux, and H. G. Viehe, Angew. Chem., Internat. Ed. 8, 454 (1969); (c) H. Eilingsfeld, M. Seefelder, and H. Weidinger, Chem. Ber. 96, 2681 (1963).

(80) I. Ugi, F. Beck, and U. Fetzer, Chem. Ber., 95, 126 (1962).

over calcium hydride overnight, and redistillation from the same hydride under nitrogen. Similar results were obtained with this rigorously dried amine, however. Discovery that the yields after 4 hr were about the same as those obtained after 15 hr of reaction suggested that phosgene was being hydrolyzed by moisture in the starting material or solvent (ether, recently opened can), and that hydrochloric acid thus generated was protonating the amide. Protonation of amides by acids has been studied before.[81] In the present case bubbling hydrogen chloride rather than phosgene into the reaction mixture for 5 min gave white crystals immediately. The nmr spectrum (No. 16) was identical to that from the previous reaction and the starting amide was again regenerated upon treatment with triethylamine. Extreme care was therefore taken to carry out the phosgene reaction under rigorously anhydrous conditions.[82] The starting material was redistilled, the solvent was changed to benzene, all the glassware was dried for at least 24 hr at 135° and assembled while hot. Under these conditions reaction of amide 59a with phosgene gas led to the formation of white crystals (60a), which, upon treatment with triethylamine, afforded 54% of N,N-dimethyl-1-chloro-2-methyl propenylamine 61a.[71,83] The nmr spectrum (No. 17) consisted of two

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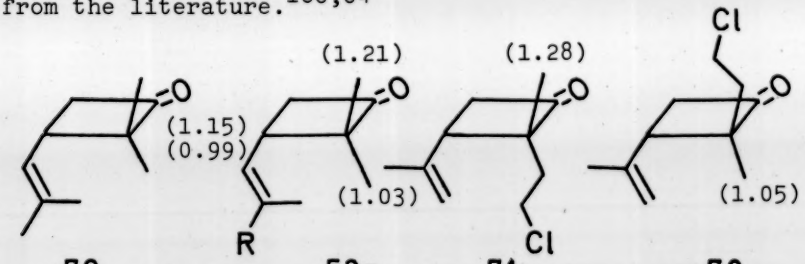
(81) (a) G. Frankel and C. Franconi, J. Am. Chem. Soc., 82, 4478 (1960); (b) G. Frankel and C. Niemann, Proc. Natl. Acad. Sci. US., 44, 688 (1958).

(82) G. W. Kramer, A. B. Levy, and M. M. Midland, in Organic Synthesis via Boranes, H. C. Brown, Ed., Wiley-Interscience, New York, 1975, Chapter 9.

(83) H. Weingarten, J. Org. Chem., 35, 3970 (1970).

singlets, as reported for this compound.[83] Treatment of this α-chloroenamine with silver tetrafluoroborate at -78° generates keteneimmonium cation 62a, in situ, which adds to diene 63 upon warming to room temperature.[71] The cyclobutyledene ammonium salt 64a readily gives cyclobutanone 53a on basic work up. Table 6 shows the difference in yields of reactions done with 12% phosgene solution and phosgene gas. It is clear that is not the reaction time, but the amount of phosgene, which determines the overall yield.

There are several characteristic features of this compound that were useful in elucidation of its structure. The infrared spectrum (No. 4) shows a strong band at 5.59μ, as expected for a 4-member ring ketone.[71,73] The nmr spectrum (No. 18) shows the cyclobutyl methyls at 1.03 and 1.21ppm,in agreement with those of model compounds drawn from the literature.[16c,84]



70 53a 71 72

Although the stereochemical assignment was not reported for analog 70,[16c] the shifts given for the methyls are close to those found for 53a. Correlation with struc-

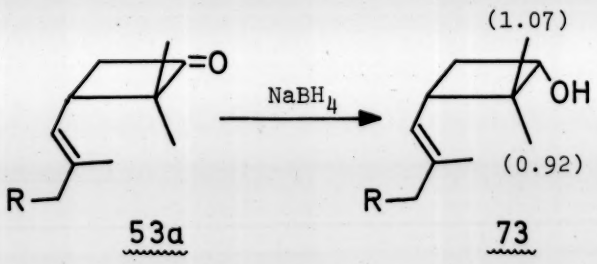
(84) J. Grandguillot and F. Rouessac, Compt. Rend. Acad. Sci., Ser. C., 277, 1253 (1973).

Table 6. Synthesis of Cyclobutanones

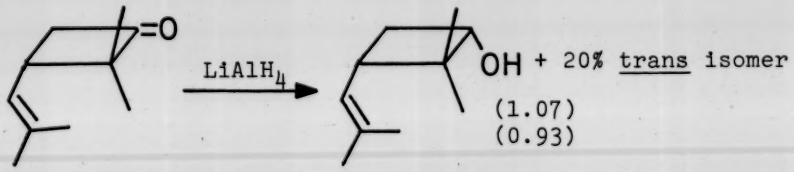
Amide (mmol)	Phosgene (mmol)	Reaction Time (hr)	α-Chloroenamine crude (mmol)	Diene (mmol)	$AgBF_4$ (mmol)	Cyclobutanone (% yield)
59a (50.00)	100[a]	30	34.3[c]	34.3		53a (15)
59a (27.35)	625[b]	21	11.66 (3)[d]	2.99	3.23	53a (59)
59b (2.22)	6.25[a]	72		excess		53b (10)
59b (8.61)	25[a]	36		excess		53b (6)
59b (15.21)	1430[b]	27	15.06 (6.94)[d]	5.85	5.85	53b (42)

[a]12% Phosgene solution. [b]Phosgene gas. [c]Not quantified. [d]Nmr quantified mmol used in next reaction.

tures 71 and 72,[84] allows unequivocal assignment of the signal at 1.21 ppm to the methyl trans to the unsaturated chain, and the signal at 1.03 ppm to the methyl cis to the chain. Confirmation of these assignments was obtained by nmr studies on alcohol (73). Cyclobutanone 53a was stereospecifically reduced with sodium borohydride to a single alcohol (73), the nmr of which also agrees with model compound 74.[16c] The stereospecific reduction can be explained by the strong steric hindrance on the more substituted face of the ring which prevents approach of the reducing agent to give the trans alcohol. Parallel stereoselectivity has been reported for the reduction of 70, which with lithium aluminum hydride gives the corresponding alcohol 74 in the ratio trans:cis, 1:4.[16c]



53a 73



70 74

A series of nmr spectra (No. 19) of cyclobutanol 73 with increasing amounts of the shift reagent $Eu(fod)_3$[36] were taken. A graph was made by plotting the shifts of the methyl signals versus the ratio of shift reagent to compound (Fig. 49). It is seen that when the ratio is 0.15, the methyl at 0.92 ppm undergoes a greater downfield shift than that at 1.07 ppm, indicating that the methyl originally at higher field is cis to the OH.[37] It must also be cis to the unsaturated chain due to the specificity of the reduction, confirming that the methyl at 1.03 ppm in 53a is cis to the unsaturated chain.

f. Attempted synthesis of 3-(2,6,10-Trimethylundeca-1(E),5(E),9-trienyl)-2-methyl-2-(4,8-dimethyl-3(E),7-nonadienyl)-cyclobutanone (53b)

The synthesis of cyclobutanone 53a was very encouraging and seemed to open a way for the preparation of 53b. The analogous synthetic sequence was therefore carried out for the preparation of 53b, without checking for formation of α-chloroenamine 61b. Tlc analysis of the crude reaction mixture after basic work up showed a major spot corresponding to starting diene, polar compounds at the origin, and two minor products. The infrared spectrum of the mixture showed a strong cyclobutanone band[73] at 5.59μ, plus strong bands at 5.83 and 6.08μ. Separation by column chromatography using silica gel gave 88% of recovered diene and a mixture of compounds with the tlc properties already described. Glc analysis showed multiple peaks with retention times as-

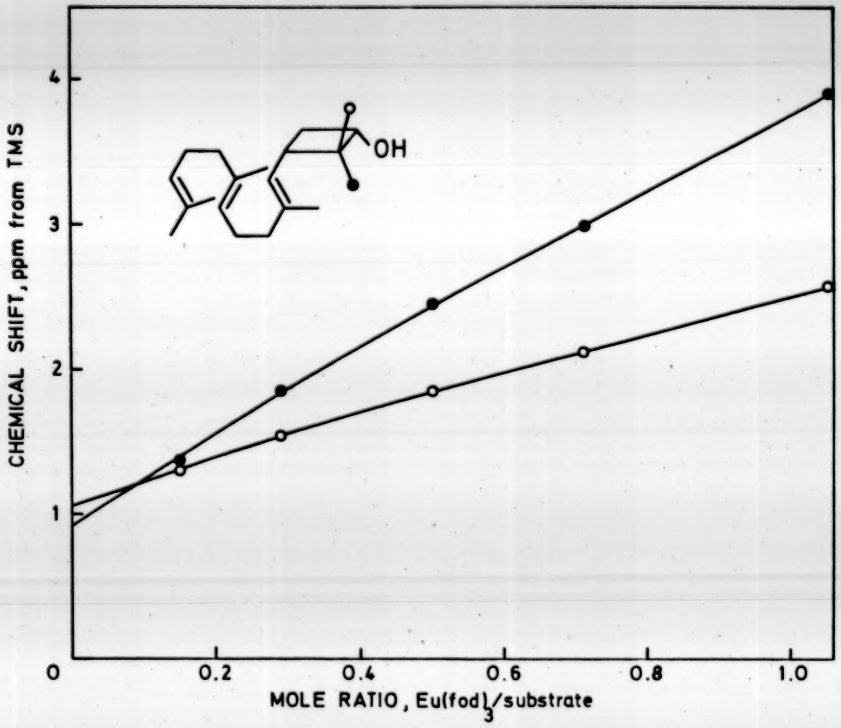


Figure 49. Variation in the chemical shift of cyclobutyl methyl groups in cyclobutanol 73 upon addition of $Eu(fod)_3$ in $CDCl_3$.

signable to C_{15} compounds, and four peaks in the C_{30} retention time region (Fig. 50A). Removal of volatiles by bulb-to-bulb distillation gave a residue that was filtered through neutral alumina to give two fractions (0.6% yield) consisting mainly of the four higher retention peaks (Fig. 50 B,C). Neither fraction exhibited the band at 5.59μ. Only bands at 5.88 and 6.08μ were present in the infrared spectrum (No. 5). On the other hand, the volatile fraction showed bands at 5.59, 5.83, and 6.08μ. These results led to the conclusion that the C_{30} compounds were not cyclobutanones. The GC-mass spectra of the four peaks are given in Fig. 51, while the nmr spectra[85] (No. 20, 21) are reproduced in the spectra section of this thesis. Stepwise analysis showed that, from the reaction of phosgene with amide 59b to give chloroiminium chloride 60b, intramolecular cyclizations were probably occurring, since the nmr spectrum (No. 22) of the salt itself did not integrate for the expected two vinylic protons, but instead only for one. Mere protonation of the amide is untenable as an explanation since the nmr spectrum shows a doublet for the N-methyls, instead of a singlet as in the protonated model compound (nmr No. 15). Intramolecular cyclization can be rationalized by considering that 60b is very susceptible to nucleophilic attack by the electron rich C_5-C_6 double bond. If indeed intramolecular cyclizations are taking place, then removal of the double bonds by hydrogenation of amide 59b should make possible the synthe-

(85) The author is grateful to Dr. S. Hurt for recording these spectra on a Varian XL-100 spectrometer.

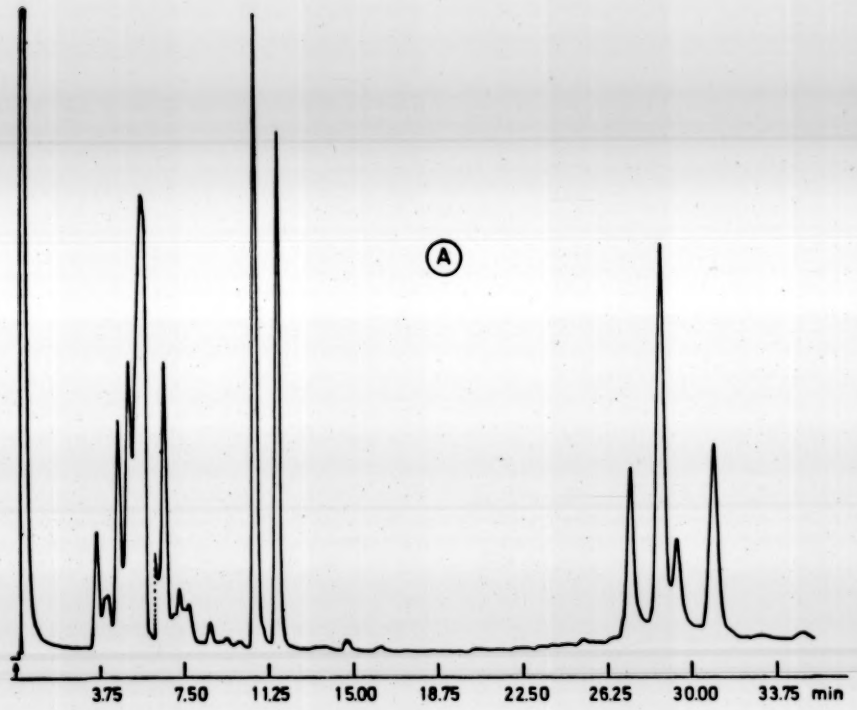


Figure 50A. GC analysis of the purified reaction mixture obtained by reaction of tetraene 63 with the product of reaction of unsaturated amide 59b, phosgene, and silver tetrafluoroborate. GC conditions: System A, programmed 150-200°, 4°/min.

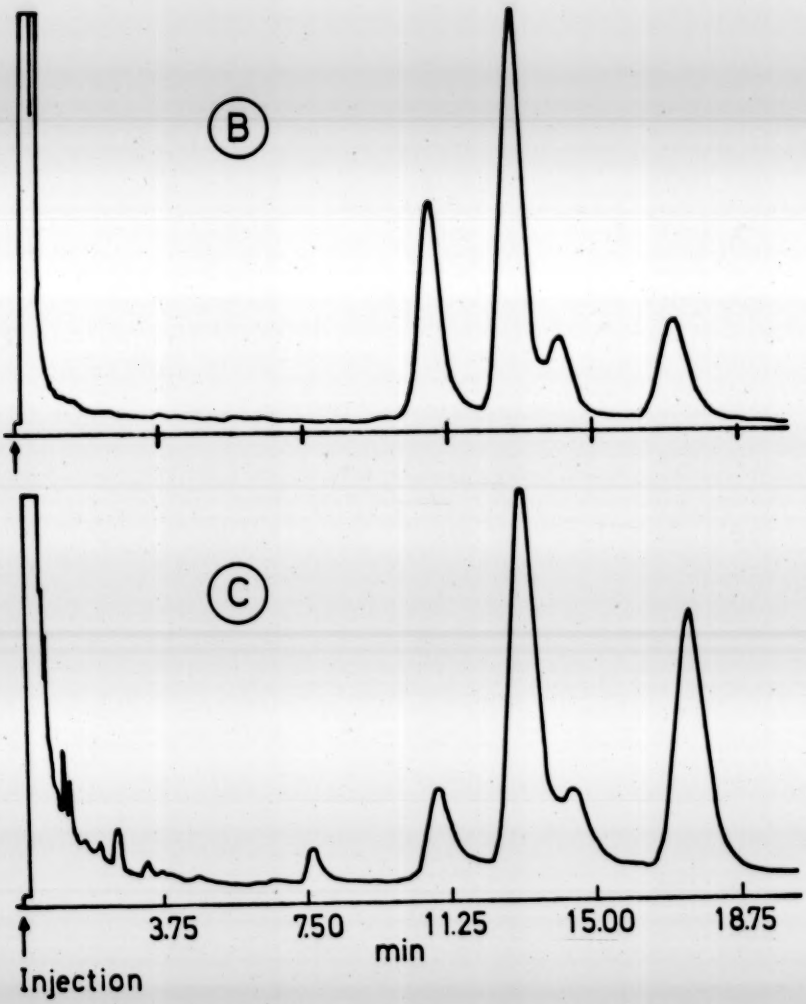


Figure 50B,C. G C analysis of the reaction mixture in Fig 50A after removal of volatiles by bulb-to-bulb distillation. G C conditions: System A, 240°

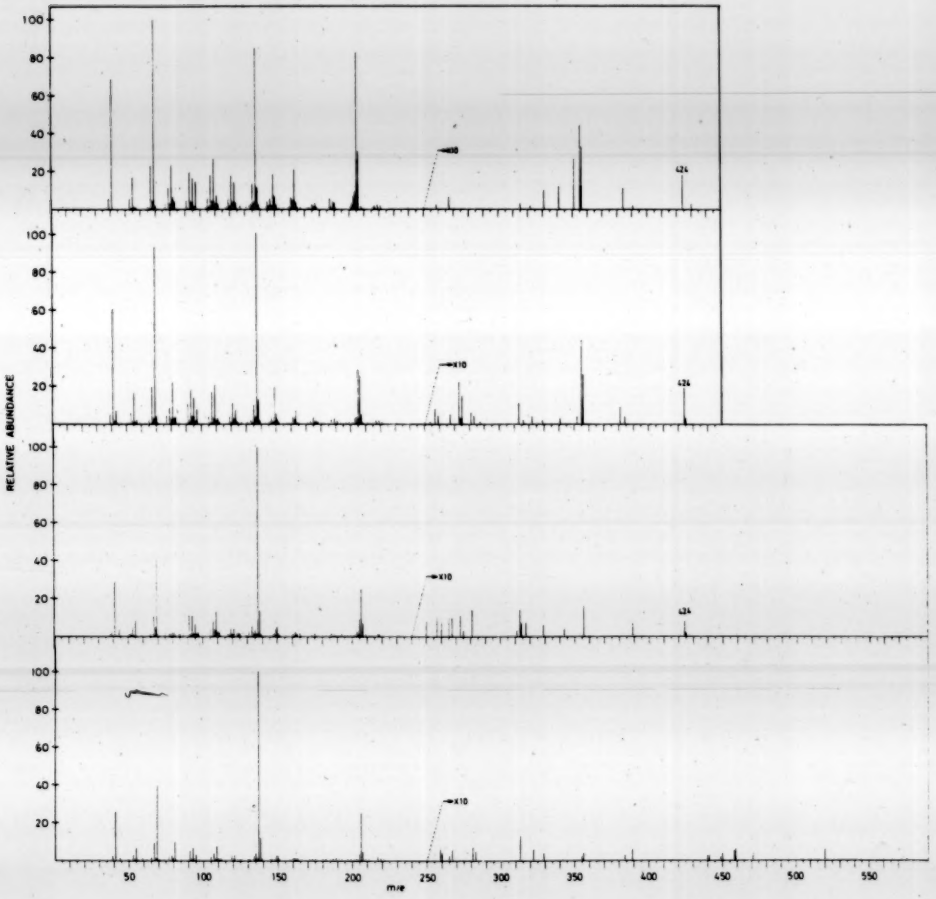
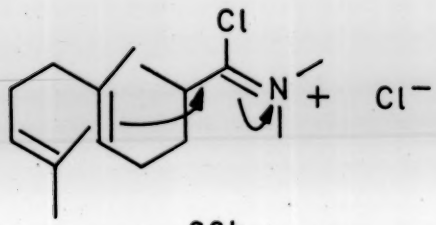


Figure 51. Mass spectra of the four components in Fig. 50B.



60b

sis of cyclobutanone 53c.

g. Synthesis of 3-(2,6,10-Trimethylundeca-1(E),5(E),9-trienyl)-2-methyl-2-(4,8-dimethyl-1-nonyl)-cyclobutanone (53c)

Amide 59c was treated with phosgene gas, to give, in quantitative yield, the corresponding α-chloroenamine 61c. The nmr spectrum (No. 23) of this compound correlates very nicely with that obtained for 61a (No. 17). Treatment of 61c with silver tetrafluoroborate in the presence of tetraene 63, as described before, afforded the expected cyclobutanone in 42% yield after isolation by preparative layer chromatography. The infrared spectrum (No.6) showed the strong cyclobutanone carbonyl band at 5.6μ. Only one spot could be detected by tlc. However, when the mixture was subjected to glc analysis two major peaks (a and b in Fig. 52 A), in the ratio a:b=64:36, were observed, together with less than 15% of unidentified impurities and volatile compounds formed by decomposition on glc. This last observation was confirmed by collecting each individual peak (a) and (b) by preparative glc and reinjecting into the analytical column. The same pat-

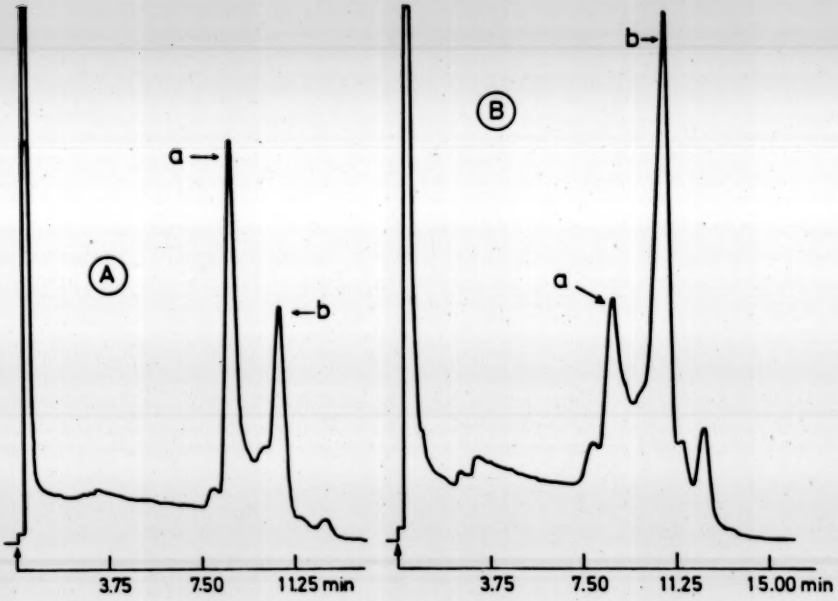
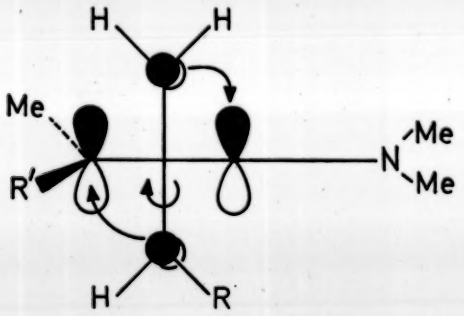


Figure 52. GC analysis of the purified reaction mixture obtained by reaction of tetraene 63 with the product of reaction of saturated amide 59c, phosgene, and silver tetrafluoroborate (A); and a fraction obtained by column chromatography of this mixture (B). GC conditions: System A, 140°.

tern of decomposition products was obtained. Assignment of stereochemistry to peaks (a) and (b) as cis and trans isomers, respectively, was achieved by correlating the cyclobutyl methyl peaks with those of model compound 53a as follows. Part of the mixture was chromatographed on silica gel to give fractions with varying concentrations of the isomers. One of these fractions contained a ratio of isomers a:b=32:68 (Fig. 52b). Its nmr spectrum (No. 25) showed one singlet at 1.03 ppm, which is the same position as the cyclobutyl methyl protons cis to the unsaturated chain in the spectrum of model compound 53a; thus, this signal corresponds to the trans isomer in the mixture. The cis isomer methyl group appears at 1.21 ppm, superimposed on the broad aliphatic methylene protons signal, in exact agreement with the cyclobutyl methyl group trans to the prenyl chain in 53a. These singlets were also observed in the nmr spectrum (No. 24) of a tlc purified reaction mixture whose ratio of isomers was approximately a:b, 64:38 (Fig. 52 A). Correlation of these nmr spectra singlets with the relative ratios of isomers (a) and (b) in (Fig. 52 A, B), shows that the singlet at 1.21 ppm increases when peak (a) increases, and the singlet at 1.03 ppm decreases when peak (b) decreases. Peak (a) is therefore the cis and peak (b) the trans isomer. When this reaction was done with 12% phosgene solution the same ratio of isomers was obtained, although the yields were lower (Table 6).

An interesting point about this reaction is the

preferential formation of the cis over the trans isomer. This observation can be explained by examining the hypothetical transition state 75 for a ($\pi^2_s+\pi^2_a$) polar cycloaddition reaction.[86] The two reacting molecules should approach in



75

such a way that the long chains are the furthest apart in order to minimize the steric interaction. The rotation during cycloaddition which generates the least steric interaction leads to the cis product.

4. Preliminary Inhibition Studies

Emulsions of cyclobutanones 53a and 53c were preincubated with enzyme for 30 min at 37°, then the normal substrate (20 μM) was added, and the incubation prolonged for 2 min. Addition of ethanol, extraction of hydrocarbon products with petroleum ether, and purification on column chromatography gave squalene that was assayed by liquid scintillation counting.

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(86) R. B. Woodward and R. Hoffmann, "The Conservation of Orbital Symmetry", Academic Press, New York, N.Y., 1969.

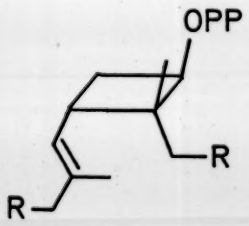
The results of these studies are given below.

Compound	Concentration Unit (mM)	1-^{3}H -1 added (μM)	Incorporation (dpm)
53a	6.0	20	7343
53c	4.5	20	7144
No inhibitor		20	7885

The poor inhibition observed in these studies (7 and 9%, respectively) excludes these compounds as full transition state inhibitors, since the latter are characterized by potent inhibition.[64] Poor inhibition could result from (1) weak binding of the analogs to the active site of the enzyme due to saturation of the double bonds in 53c, and lack of the hydrocarbon chain in 53a; (2) low concentration of these compounds at the active site due to their poor solubility in the aqueous incubation mixture employed or (3) the compounds are bound and do not act as inhibitors. The effect of the double bonds and length of the chain in binding is emphasized by the weak inhibition produced by saturated farnesyl pyrophosphate analog 22 and short geranyl pyrophosphate 21. Analog 53b thus is still of interest as the most proper for evaluation as a transition state inhibitor.

Cyclobutyl pyrophosphate 54 was prepared by the general procedure described before (Part A), and purified by column chromatography using silica gel. Inhibition studies

showed that this compound is as weak an inhibitor of squalene synthetase as geranyl pyrophosphate 21. This observation is not unexpected if one considers that the hypothetical intermediate[9e] (76) in the rearrangement of presqualene pyrophosphate has the pyrophosphate group trans to the hydrocarbon chain, whereas in 54 the pyrophosphate moiety is cis to the prenyl chain.



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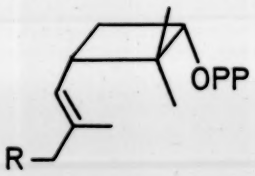
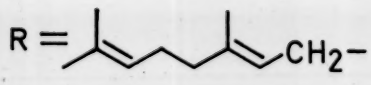


54



EXPERIMENTAL

Instrumentation. Infrared spectra were obtained as thin films using a Perkin-Elmer Model 337 grating spectrophotometer with polystyrene as a calibration standard, values are given in microns (μ). Nmr spectra were taken on a Varian A-60A spectrometer as approximately 25% v/v deuterochloroform solutions. Signals are reported in parts per million (ppm) downfield from the internal standard tetramethylsilane. The nmr shift reagent $Eu(fod)_3$ was obtained from Willow Brook Laboratories and was always transferred under a nitrogen atmosphere. Optical densities were measured with a Carl Zeiss PMQ II spectrophotometer. Mass spectra were taken on an AEI-MS-9 adapted to a chemical ionization mode (isobutane gas). Glc-mass spectrometric analyses were carried out on an AEI-MS-12 instrument equipped with a Biemann-Watson molecular separator and an Infotronics Model 2400 gas chromatograph fitted with 6ft x 2.5mm i.d. columns. The stationary phase was 2% Dexsil 300 on 80-100 chromosorb GHP. Radioactivity measurements were made by liquid scintillation counting on a Packard Tri-Carb Model 337S spectrometer, having an efficiency of about 40% for tritium. The amount of radioactivity in the samples was determined after addition of New England Aquasol (10ml). Low speed centrifugation was done with a refrigerated Sorvall RC-2 centrifuge, while high speed centrifugation was carried out on a refrigerated Beckman Spinco Model L vacuum ultracentrifuge. Incubations were performed in an

Eberbach constant temperature bath. Gas liquid chromatography was done in one of the following systems:

A) Varian 2100, N_2 carrier at 18 ml/min, flame ionization detector, 6 ft x 2 mm i.d. (glass column), 3% OV-225 on 100-200 mesh Varaport 30.

B) Varian 2100, N_2 carrier at 28 ml/min, flame ionization detector, 6 ft x 2 mm i.d. (glass column), Carbowax 20 M.

C) Varian 2100, N_2 carrier at 18 ml/min, flame ionization detector, 6 ft x 2 mm i.d. (glass column), 2.5% Dexsil 300 on 80-100 Chromosorb GHP, oven temperature at 260°.

D) Varian 2100, N_2 carrier at 26.5 ml/min, flame ionization detector, 6 ft x 2 mm i.d. (glass column), 2% OV-225 on Varaport 30.

E) Varian 90-P, He_2 carrier at 60 ml/min, thermal conductivity detector, 10 ft x 0.25 inch i.d. (steel column), 3% OV-225 on 100-120 Chromosorb W.

Bulb-to-bulb distillations were carried out on a Buchi Kugelrohr. All spinning band distillations were done on a Nester Faust 19 inch teflon spinning band column. The fractions were collected in tared vials and analyzed by glc.

Thin Layer and Column Chromatography. General analytical thin layer chromatography was performed on commercial (Analtech) glass plates coated with silica gel GF 254 (2.5 x 10 cm, 250 microns) preconditioned by storage in a desiccator over silica gel for at least 24 hr. Organic compounds were visualized by UV illumination, by exposure to iodine

vapor, or by charring after spraying with 75% aqueous sulfuric acid. Purification of pyrophosphates was monitored with Eastman Kodak (No. 1381) plastic-backed silica gel plates with fluorescent indicator. Preparative layer chromatography was done on commercially available 20 x 20 cm Merk silica gel PF 254 plates (2 mm thickness). Compound visualization was achieved by UV light or partial development with iodine vapors. Column chromatographic separations were done on either Merk silica gel 60 (70-230 mesh) or basic alumina grade II.

Solvents. Unless otherwise stated, reagent grade solvents were purified and dried before use. The following solvents were purified as indicated: tetrahydrofuran and ether were distilled from lithium aluminum hydride after refluxing for 2-3 hr; hexamethylphosphoramide was stirred overnight with sodium under nitrogen and distilled *in vacuo*; hexane, petroleum ether, dichloromethane, acetonitrile, and trichloroacetonitrile were distilled from phosphorus pentoxide after refluxing for 1-2 hr; benzene was distilled from sodium after stirring for 2-3 hr; triethylamine was distilled from calcium hydride, the distillate refluxed overnight over more of the same hydride, and finally distilled under nitrogen.

General Reaction Procedures. All reactions, unless specified otherwise, were carried out under a nitrogen atmosphere in glassware dried overnight in an oven at 135°. Addition of liquid reagents was done with a dry hypodermic syringe through rubber septa attached with a rubber band

onto a neck of the reaction flask. Solid reagents were added via a neck of the reaction flask through which a steady stream of nitrogen was flowing. Reactions were monitored either by thin layer chromatography or gas-liquid chromatography. Organic extracts of reaction mixtures were dried over anhydrous magnesium sulfate unless noted otherwise. The dried extracts were concentrated by evaporation at 25-50° on a rotatory evaporator evacuated to 10-20 mm (trap cooled with dry ice-acetone) by water aspirator. Temperatures are given in Centigrade degrees and are not corrected.

Microanalyses. Microanalyses were performed by the University of California Microanalytical Laboratory, Berkeley.

Abbreviations. THF, tetrahydrofuran; DMF, dimethylformamide; HMPA, hexamethylphosphoramide; ether, diethyl ether; pet-ether, petroleum ether; NADPH, nicotinamide adenine dinucleotide phosphate, reduced form; CH_2Cl_2, dichloromethane; CH_3CN, acetonitrile; $Eu(fod)_3$, tris(1,1,1,2,2,3,3-heptafluoro-7,7-dimethyl-4,6-octanedionate)-europium (III); nmr, nuclear magnetic resonance (b=broad, s=singlet, d=doublet, t=triplet, q=quartet, m=multiplet); ir, infrared (v=very, s=strong, m=moderate, w=weak, sh=shoulder, wm=weak moderate); bp, boiling point; glc, gas-liquid chromatography (tr=retention time); CIMS, chemical ionization mass spectrum; tlc, thin layer chromatography (Rf=center of mobility of compound relative to solvent front); Anal., elemental analysis; cpm, counts per minute;

dpm, decompositions per minute (cpm/efficiency).

Synthesis of Pyrophosphates.[22] Di-triethylammonium phosphate (450 mg, 1.5 mmol) was dissolved in 30 ml of dry CH_3CN (warming was neccesary), and added over 4 hr at 25° to a stirred solution of the farnesol analog (0.5 mmol) and trichloroacetonitrile (650 mg, 4.5 mmol) in 5 ml of CH_3CN. The mixture was stirred for 24 hr at 25° and concentrated *in vacuo*. The dark yellow residue obtained was transferred with 10 ml of acetone to a centrifuge tube and treated with concentrated ammonia (0.5-1 ml). The precipitated ammonium salts, isolated by centrifugation, were washed twice by resuspension in 5 ml aliquots of acetone containing 0.01 N NH_4OH. Purification of the crude reaction mixture was done as follows:

Procedure A. The residual white solid was dissolved in 5 ml of 0.01 N aqueous ammonia and added to 100 g of prewashed Amberlite XAD-2 resin[22] suspended in 100 ml of 0.01 N aqueous ammonia. The resin, collected on a sintered funnel after stirring overnight, was washed with 0.01 N aqueous ammonia (5 x 60 ml). The organic monophosphate and pyrophosphate were eluted from the resin with methanol containing 0.01 N ammonia (5 x 60 ml). The methanol extracts were combined and concentrated *in vacuo* at 30-50°. The residue was transferred with 2 ml of methanol containing 0.01 N NH_4OH to a tared centrifuge tube. Addition of acetone containing 0.01 N NH_4OH (4-8 ml) precipitated the pyrophosphates with small amounts of monophosphates which

after centrifugation, were removed by a second or third precipitation. The purified pyrophosphates were dried overnight at 25° under high vacuum.

Procedure B. The white solid from cyclobutyl alcohol 54 (0.5 mmol) was chromatographed on preconditioned silica gel[18,39] (10 g, 21 x 1 cm column). This column was washed with 70 ml of n-propanol-NH_4OH-H_2O, 6:1:1. The crude product was then added in 2 ml of the same solvent. The column was eluted with 100 ml of the solvent above, collecting 1 ml fractions. Elution of monophosphate coincided with elution of a yellow band (fractions 14-16), while fractions 18-24 precipitated pure pyrophosphate upon addition of acetone (2 ml). The combined pyrophosphate fractions were centrifuged, separated from supernatant, and dried overnight under high vacuum to yield 4 mg (4.8%) of pure pyrophosphate 54. Tlc analysis (n-propanol-NH_4OH-H_2O, 6:3:1) showed one spot (Rf 0.61). In the same system the monophosphate had Rf 0.64.

The purity of the fractions obtained by either procedure was assayed on silica gel plates. Monophosphates appear as blue spots and pyrophosphates as purple spots on visualization with Rosenberg's reagent.[87]

Phosphorus Determination.[40] Into a test tube containing a solution of the pyrophosphate analog (about 0.05 μmol/ 0.2-2 ml) was added 0.5 ml 10N H_2SO_4, and the mixture heated

(87) H. Rosenberg, J. Chromat., 2, 487 (1959).

in an oven at 150-160° for 3 hr. Then, 2 drops of 30% H_2O_2 were added at 25° and heated again for 1.5 hr in order to complete combustion and decompose excess peroxide. The colorless solution was diluted with 4.4 ml H_2O, treated with 0.2 ml of 5% ammonium molybdate and 0.2 ml of the Fiske-SubbaRou reagent,[40] mixed thoroughly, and heated in an oil bath at 100° for 7 min. Optical densities were measured at 830 mμ and the phosphorus content obtained from a standard plot of optical densities vs μ moles of phosphorus. This graph in turn was prepared with standard solutions containing 0.00, 0.02, 0.04, 0.06, 0.08, 0.10, 0.12, 0.15, and 0.20 μ mol of phosphorus (KH_2PO_4).

Alkaline Phosphatase Hydrolysis.[7] The pyrophosphate analog (about 0.4 μM) was incubated with a 10 ml mixture containing: tris HCl (PH 8.1), 1 mM; $MgCl_2$, 0.05 mM; alkaline phosphatase 0.4 μM; water, 8 ml. After 20 min at 25°, the mixture was extracted with pet-ether or hexane. The extracts were concentrated in vacuo, transferred to a conical tube, and concentrated under a nitrogen stream. The hydrolysis product was analyzed by tlc and glc.

E , E -Farnesol. Farnesol (practical grade) obtained from Fluka A G (Switzerland) was shown by glc analysis (System A, 150°) to be a 1:2 mixture of the Z , E (tr 13.13 min) and E , E (tr 15.03 min) isomers. An 88 g sample was distilled continuously through a teflon spinning

band column[88] maintaining a reflux ratio of about ten to one and a reflux rate of about 20 drops/min. The results are given below.

Fraction	Weight (g)	% all-(E)	Fraction	Weight (g)	% all-(E)
1	3.10		11	2.29	75
2	7.61		12	2.31	81
3	1.32	10	13	2.45	83
4	1.97	15	14	3.84	89
5	2.12	50	15	3.24	93
6	3.40	50	16	1.76	96
7	11.95	70	17	1.96	100
8	5.66	40	18	1.29	100
9	2.23	70	19	3.59	100
10	2.70	70	Residue	16.69	

E,E-Farnesal. This compound was prepared from 4.3 mmol of E,E-farnesol and 115 mmol of activated manganese dioxide[89] following the procedure described for the preparation of 3-desmethylfarnesal 46. Filtration of the reaction mixture, washing well with pet-ether, concentration in vacuo and bulb-to-bulb distillation (90°/.075 mm) yielded pure

(88) P. R. Ortiz de Montellano, Ph. D. Thesis, Harvard University, 1968, p. 80.

(89) J. Attenburrow, A.F.B. Cameron, J. H. Chapman, R.M. Evans, B.A. Hems, A.B.A. Jansen, and T. Walker, J. Chem. Soc., 1094 (1952).

E,E-farnesal (3.47 mmol, 81%) as a light green oil: ir 5.93 (s, C=O), and 6.10 (m,C=C); nmr 1.62 (s, 6H, C(7,11)CH_3), 1.68 (s,3H,C(11)CH_3), 1.93-2.37 (m,8H, CH_2C=), 2.17 (d, J=1.5Hz, 3H, C(3)CH_3), 5.13 (bs, 2H, HC=), 5.92 (d, J=8Hz, 2H, C(2)H), and 10.05 (d, J=8Hz, 1H, C(1)H). Tlc analysis showed one spot (Rf 0.42, 10% ethyl acetate in hexane); glc analysis showed one peak (system A, 160°, tr 14.7 min).

E,E-Methyl farnesoate. Sodium cyanide (1.15 g, 23.5 mmol) was dissolved in a solution of E,E-farnesal (0.8 g, 3.64 mmol) and acetic acid (0.41 g, 6.84 mmol) in 25 ml of dry methanol at 25°. To this solution was added 10 g (103.5 mmol) of activated manganese dioxide and the resulting mixture was stirred 14 hr at 25°.[50] Vacuum filtration and concentration in vacuo gave a residue that was worked up in water (10 ml) and ether (3 x 20 ml) yielding 0.75 g of crude product. Tlc analysis (8% ethyl acetate in hexane) showed a major spot (Rf 0.50), some recovered aldehyde (Rf 0.26) and two minor contaminants (Rf 0.56 and 0.15). This mixture was chromatographed on silica gel (30g, 2.5 x 20 cm column) using benzene-chloroform (97:3) as eluting solvent. The fractions with Rf 0.50 in the system above were combined and bulb-to-bulb distilled (88°,0.25mm) to afford 559 mg of impure methyl farnesoate divided between two fractions (A and B). Glc analysis (system E, 160°) showed two peaks (1, tr 10.48 min; 2, tr 12.28 min). Fraction A consisted of components 1 and 2 in the ratio

5:95, respectively. These components were present in fraction B in the ratio 22:78. Fraction A was employed in the preparation of radiolabeled farnesol. The infrared spectrum showed a strong absorption at 5.76 (C=O) and a medium one at 6.01 (C=C); nmr[90] 1.59 (s,6H, C(7,11)CH_3), 1.66 (s, 3H, C(11)CH_3), 1.93-2.33 (m, 8H, CH_2C=), 2.14 (d, J=1.5Hz, C(3) CH_3), 3.62 (s, 3H, CH_3O), 5.03 (bs, 2H, HC=) and 5.6 (s, 1H, C(2)H).

1-H^3 - E,E-Farnesol.[91] To a stirred suspension of lithium aluminum hydride (5.4 mg, 0.1425 mmol) in 3 ml of dry ether was added a solution of E,E-farnesal (140.5 mg, 0.6376 mmol) in 1 ml of dry ether at 0° under a nitrogen atmosphere. Tlc analysis of an aliquot showed some product formation after 30 min stirring at room temperature. At this time tritium labeled lithium aluminum hydride (1.42 mg, 0.0375 mmol , specific activity 130 mC/mmol) was added at 0°. The reaction mixture was stirred for 2 hr at room temperature and treated with lithium aluminum hydride (1 mg, 0.0264 mmol) at 0°. After 90 min stirring at 25°, tlc analysis showed no starting material, and the excess hydride was decomposed by addition of water (1 equivalent) at 0°. The mixture was stirred for 15 min, filtered through a sintered funnel, and the residue washed well with ether.

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(90) R. J. Anderson, C. A. Henrick, J. B. Siddall, and R. Zurflüh, J. Am. Chem. Soc., 94, 5379 (1972).

(91) (a) I. Shechter and K. Bloch, J. Biol. Chem., 246, 7690 (1971); (b) 22

The filtrate solution was concentrated *in vacuo* to give 141.7 mg (100%) of labeled product (specific activity 3 mC / mmol). This product was found to cochromatograph on tlc (Rf 0.28; 10:1, benzene-ethyl acetate) with an authentic sample of unlabeled E,E-farnesol.

E , E -Ethyl farnesoate.[22,90] Sodium hydride (17.28 g of a 50% oil suspension, 0.36 mol) washed with pet-ether, was stirred in 600 ml of dry THF under nitrogen atmosphere. Triethyl phosphonoacetate (106.1 g, 0.47 mol) in 100 ml of dry THF was added via syringe at 0° over a 30 min period. The mixture was stirred at 30-40° until hydrogen evolution ceased (1 hr). After cooling to 0°, 70 g (0.36 mol) of (E)-geranylacetone[29] was added in 15 min, and the mixture stirred for 16 hr at 25°. Water (100 ml) was added, the layers separated, and the aqueous layer extracted with pet-ether. The organic layer was concentrated *in vacuo* and dissolved in 300 ml of pet-ether. The combined pet-ether extracts were washed with water, dried, filtered and concentrated *in vacuo* to give 94.6 g of crude reaction mixture. Glc analysis (system A, 150°) showed 0.66% of recovered geranylacetone (tr 4.31 min), and 95% of ethyl farnesoate (tr 2 Z , 13 min; 2 E , 17 min) with the ratio 19:81, respectively.

Slow distillation through a short Vigreux column gave the following fractions.

Fraction	B p at 0.025 mm	Weight (g)	% All-(E)
1	21-64	1.56	
2	64-90	9.97	70
3	87	5.58	68
4	86.5	22.64	71
5	84	23.25	78
6	84-94	12.81	88
7	94	11.23	92
8	94-96	5.84	96
Residue		2.78	

The pot residue was bulb-to-bulb distilled to give 1.91 g of ethyl farnesoate (2 Z /2 E =1/99): ir 5.80 (s, C=O) and 6.02 (s,C=C); nmr[90] 1.25 (t, J=7Hz, 3H, CH_3CH_2), 1.60(s, 6H, C(7,11)CH_3), 1.68 (s, 3H, C(11)CH_3), 1.83-2.38 (m, 8H, CH_2C=), 2.18 (d, J=1 Hz, 3H, C(3)CH_3), 4.16(q, J=7Hz, CH_3CH_2), 5.12 (bs, 2H, vinyl H), 5.7 (s, 1H, C(2)H). Tlc analysis (5% ethyl acetate in hexane, two developments) showed one spot (Rf 0.40); glc analysis (system A, 150°) showed the ratio 2Z:2E, 1:99 at 13 and 17 min, respectively.

Ethyl-3,7,11-Trimethyl dodecanoate (35). Ethyl farnesoate (3.055 g, 11.55 mmol) in dry ethanol (100 ml) was hydrogenated over 10% palladium-on-charcoal (150 mg) at 25° and 1 atm. The mixture was separated by suction filtration using a sintered funnel with a celite pad, and the residue washed several times with ethanol. Concentration in vacuo and bulb-to-bulb distillation (82°/0.075 mm) gave 3.11 g (99%) of pure ester 35: ir 5.75 (s, C=O), 6.80 (s), 7.25 (s), 8.00 (w), 8.40 (s), 8.55 (s), 8.85 (m), 9.12 (w), and 9.66 (s); nmr 0.87 (d, J=6 Hz, 9H, CH_3 on C(7,11)), 0.95 (d, J=6 Hz, 3H, CH_3 on C(3)), 1.05-1.46 (m, 12H, CH_2), 1.25 (t, J=7 Hz, 3H, CH_3CH_2O), 1.25-2.47 (m, 5H C(2)H, C(3,7,11) H), and 4.13 (q, J=7 Hz, 2H, CH_3CH_2O). Tlc analysis showed one spot (Rf 0.48; 5% ethyl acetate in hexane); glc analysis showed a single peak (tr 6.11 min; System A, 150°) CIMS m/e 271 (MH^+).

Anal. Calcd for $C_{17}H_{34}O_2$: C, 75.50%; H, 12.67%. Found: C, 75.62%; H, 12.74%

3,7,11-Trimethyl dodecan-1-ol (34). Into a stirred solution of ethyl-3,7,11-trimethyl dodecanoate (1 g, 3.7 mmol) in 10 ml of dry THF was injected sodium bis-(2-methoxyethoxy) aluminum hydride (1.05 ml of a 3.58 M solution in benzene, 3.76 mmol) at 0° under nitrogen, and the mixture stirred at 25° for 3 hr. Addition of water at 0° until precipitation occurred, filtration, solvent removal and bulb-to-bulb distillation (95°, 0.075 mm) gave pure alcohol 34[26] as a colorless liquid (0.78 g, 92%): ir 3.02 (vs, OH), 9.52 (s, C-O);

nmr (No. 3) 0.87 (d, J=6Hz, 12H, CH_3), 1.05-2.00 (m, 17H, CH_2, CH), 2.70 (broad s, 1H, OH), and 3.67 (t, J=7Hz, 2H, CH_2O). Tlc analysis showed one spot (Rf 0.30, 10:1, benzene-ethyl acetate); glc analysis showed a single peak (tr 7.01 min; System A, 150°). CIMS m/e 229 (MH^+).

Anal. Calcd for $C_{15}H_{32}O$; C, 78.87%; H, 14.12%. Found: C, 78.85%; H, 14.14%.

7,11-Dimethyl-3-iodo-2(E),6(E),10-dodecatrien-1-ol(32). Into a stirred suspension of $LiAlH_4$ (34 mg, 0.91 mmol) and sodium methoxide (prepared from 1.06 mmol of sodium in anhydrous methanol) in 10 ml of anhydrous THF was added dropwise a solution of 7,11-dimethyl-6(E),10-dodecatrien-2-yn-1-ol (30)[23] (250 mg 1.215 mmol) in 5 ml of anhydrous THF at 25° with subsequent gas evolution. The mixture was stirred for 10 min and refluxed under nitrogen for 3 hr. The complex salt formed[24] was treated with excess iodine (2.79 g, 10.935 mmol) , dissolved in 20 ml of THF , for 30 min at -78°. Excess iodine was removed at 0° by addition of a basic thiosulfate solution (1 M $NA_2S_2O_3$, 2.2 M NaOH) until the solution became clear. The layers were separated and the aqueous phase extracted with ether (3 x 30 ml). The combined organic extracts were washed with water twice and dried over sodium sulfate-magnesium sulfate. Concentration in vacuo left a yellow residue which was chromatographed on basic alumina grade II (10 g, 7 x 1 cm column) eluting with hexane (30 ml), hexane-ethyl acetate 94:6 (50 ml).The

fractions collected and analyzed by tlc (hexane-ethyl acetate 65:35) were in all cases a mixture containing a major spot (Rf 0.57) contaminated with a minor component (0.49). These fractions were combined for a total of 0.2528 g (62%). The reaction was scaled up two-fold and repeated. The crude product of this reaction was chromatographed on basic alumina grade II (70 g, 2 cm id column) and eluted with petroleum ether (10 ml) and petroleum ether-ether, 5:1 (200 ml). The purity of these fractions was similar to that from the first reaction. Concentration of these combined fractions gave 0.5802 g (72%) of a light yellow oil. The purified products from both reactions were mixed. Glc analysis (6 ft 3% OV-225 glass column, 165°, 26 ml/min) showed the components in the ratio and retention times given below. Volatile

Component	Retention time (min)	% in total mixture
Unidentified	0.50	2
"	1.65	3
"	8.40	4
"	9.50	5
3-iodo compound 32	10.80	86

compounds were removed with high vacuum (125°/0.6 mm). Ir 3.00 (vs, OH) and 6.01 (w, C=C); nmr (No.1) 1.6 (s, 6H, C(7, 11)CH_3), 1.67 (s, 3H, C(11)CH_3), 1.9-2.7 (m, 8H, CH_2C=), 4.11 (d, J=5.5 Hz, 2H, C(1)H), 5.03 (m, 2H, vinyl H) and 5.5 (t, J=5.5 Hz, 1H, C(2)H).

7,11-Dimethyl-2-iodo-2(E),6(E),10-dodecatrien-1-ol (33). Into a stirred solution of propargylic alcohol (30)[23] (1.568 g, 7.6 mmol) in 7 ml of anhydrous ether was added 7.6 mmol of n-butyllithium (3.46 ml of a 2.2 M solution in hexane) followed by 22.8 mmol of diisobutyl aluminum hydride (4.5 ml of a 20% solution in hexane) and the mixture warmed for 48 hr at 35°. Excess hydride was decomposed by addition of ethyl acetate (1.5 ml, 15 mmol) at 0°. The resulting solution was treated with excess iodine (17.3 g, 68.4 mmol) in 100 ml of ether for 10-15 min at -78°. The reaction mixture was poured into basic sodium thiosulfate solution (30 g $Na_2S_2O_3$ and 11 g NaOH in 120 ml H_2O), extracted with ether and dried. After evaporation of solvents in vacuo the crude product was dissolved in 2 ml of petroleum ether and chromatographed on basic alumina II (70 g, 40 x 2 cm column). The column was eluted with pet-ether (200 ml), 5:1 petroleum ether-ether (120 ml), and ether (200 ml). Evaporation of the ether fraction left 0.56 g (21.8%) of alcohol 33[23,92] as a pale yellow oil: ir 3.00 (vs, OH) and 6.00 (w,C=C); nmr (No.2) 1.65 (s, 6H, C(7,11)CH_3),

(92) In an attempt to prepare the title compound by the procedure described for the preparation of 32, 2.425 mmol of 30 in a suspension of 6.275 mmol of $LiAlH_4$ and 0.1046 mmol of $AlCl_3$ in 40 ml THF were mixed and refluxed for 3 hr. Addition of excess iodine (21.87 mmol) at -78°, followed by treatment with basic sodium thiosulfate, extraction, and chromatography of the concentrated extract afforded 1.88 mmol (78%)of a light yellow oil which had identical physical properties (Rf, ir, nmr) as 3-iodo compound 32.

1.72 (s, 3H, C(11) CH_3), 1.97-2.40 (m, 8H, CH_2C=), 2.8 (bs, 1H, OH), 4.22 (s, 2H, C(1) H), 5.13 (bs, 2H, vinyl H), and 5.9 (m, 1H, C(3)H). Tlc analysis showed one spot (benzene-ethyl acetate 10:1, Rf 0.44); glc analysis (System A, 175°) showed a major peak (tr 21.94 min) contaminated with less than 3% of unidentified volatile compounds.

Ethyl-2,3,7,11-Tetramethyl-2(E),6(E),10-dodecatrienoate (37). This compound was prepared from 86 mmol of sodium hydride, 87 mmol of diethyl 1-carboethoxyethyl phosphonate,[27] and 86 mmol of E -geranylacetone[29] following the procedure described for the preparation of ethyl farnesoate. The mixture was stirred for 24 hr at 60°. The crude product was distilled through a short Vigreux column to give 4.88 g of geranylacetone, and 14.30 g (84% adjusted yield) of a 1:1 mixture of 2E and 2Z ethyl ester 37 (bp 110°, 0.15 mm). The isomeric mixture was subjected to spinning band distillation maintaining a reflux ratio of 15:1 and a reflux rate of about 17 drops/min for a period of 24 hr. The fraction number, weight in grams, and ratio 2Z:2E are given, respectively, as follows: 1, 1.97, 74:26; 2, 2.57, 88:12; 3, 1.55, 93:7; 4, 2.33, 61:39; 5, 1.17, 33:67; 6, 0.90, 16:84; and 7, 0.79, 11:89. Filtration of the remaining pot residue through basic alumina (grade II, 20g, pet-ether solvent), concentration in vacuo and bulb-to-bulb distillation (85°/0.050 mm) furnished 2.5 g of

ethyl-2-methyl farnesoate 37 (2Z/2E, 2/98): ir 5.78 (s, C=O), 6.02 (m, C=C), 6.83 (s), 7.17 (m), 7.22 (sh), 7.75 (sh), 7.86 (m), 8.31 (s, C-O), 9.15 (s, C-O), 9.73 (w), and 12.95 (m); nmr (No. 4) 1.28 (t, J=7 Hz, 3H, CH_3CH_2O), 1.60 (s, 6H, C(7,11) CH_3), 1.66 (s, 3H, C(11)CH_3), 1.87 (s, 3H, C(2) CH_3), 1.76-2.23 (m, 11H, CH_2C=, C(3)CH_3), 4.18 (q, J=7 Hz, 2H, CH_2O), and 5.15 (bs, 2H, CH=C). Tlc analysis showed a single spot (Rf 0.36; 5% ethyl acetate in hexane). Glc analysis showed two peaks in the ratio described previously (System A, 150°; 2Z, 17 min; 2E, 21 min). The CIMS showed a base peak m/e 279 corresponding to $C_{18}H_{30}O_2 + H^+$ and salient peaks at m/e 251 ($MH^+ - 28$) and 233 ($MH^+ - 46$).

Anal. Calcd for $C_{18}H_{30}O_2$: C, 77.65%; H, 10.86%. Found: C, 77.33%; H, 10.76%.

2,3,7,11-Tetramethyl-2(E),6(E),10-dodecatrien-1-ol (36). A solution of 37 (1.5 g, 5.39 mmol) in 15 ml of dry ether was injected into a stirred suspension of lithium aluminum hydride (256 mg, 6.75 mmol) in 20 ml of ether at 0°. After being stirred at 25° for 1.5 hr, the reaction mixture was treated with water (0.49 ml, 27 mmol) at 0°. The resulting slurry was stirred 5 min and filtered through a sintered funnel, washing the residue with ether.

Concentration in vacuo and bulb-to-bulb distillation (95°, 0.075mm) afforded pure alcohol 36 as a colorless mobile liquid (1.1 g, 86%): ir 3.03 (vs, OH), 5.99 (w, C=C), 6.85 (m), 7.27 (m), 8.13 (w), 8.66(w), 9.05 (w), 10 (s, C-O), 11.24 (w), and 11.98 (w); nmr (No.6) 1.61 and 1.69 (singlets, 12H, allyl CH_3), 1.74 (s, 3H, C(2) CH_3), 1.83 (s, 1H, D_2O exchange, OH), 1.93-2.2 (m, 8H, allyl CH_2), 4.12 (s, 2H, CH_2O) and 5.61 (bs, 2H, vinyl H). Tlc analysis showed a single spot (Rf 0.29; 10:1, benzene-ethyl acetate). Glc analysis showed a major peak, 98%, (tr 20.29 min; system A, 150°). The CIMS showed a parent ion peak at m/e 219 ($MH^+ - H_2O$) and salient peak at m/e 205 ($MH^+ - CH_3OH$).

Anal Calcd for $C_{16}H_{28}O$: C, 81.29%; H, 11.94%. Found: C, 81.10%; H, 11.80%.

7,11-Dimethyl-6(E),10-dodecadien-3-one (39). Ethyl 3-ketopentanoate (30 g, 0.205 mol) was added dropwise at 42-48° to a stirred solution of sodium ethoxide prepared from 4.72 g of sodium and 150 ml of anhydrous ethanol. The resulting solution, cooled to ambient temperature, was added with stirring to 64 g (0.295 mol) of geranyl bromide[33] in 40 ml of ethanol at -7°. The mixture was stirred 30 min at -4° and 3 hr at 25°. Concentration in vacuo gave a yellow oil which was hydrolyzed by stirring in 100 ml of 13% aqueous sodium hydroxide for 2 hr at 80°. The oil layer, combined with a benzene extract of the aqueous layer, was washed with water, dried, and freed of solvent, yielding 39.36 g of a

light yellow oil. Fractional distillation through a short Vigreux column gave the four fractions below:

Fraction	Bp/0.15 mm	Weight (g)
1	25-47	4.5
2	47-70	2.7
3	70-80	14.3
4	80-90	3.8
Residue		13.0

Further hydrolysis of the residue for 24 hr gave 3.8 g of the fraction distilling at 80-90°/0.15 mm. This fraction, together with 3 and 4 from above were redistilled to give 18 g (42%) of ketone 39 as a clear liquid: ir 5.80 (s, C=O), 5.95 (w, C=C); nmr 1.03 (t, J= 7.5Hz, 3 H, $\underline{CH_3}CH_2$), 1.59 (s, 6H, C(7,11) CH_3), 1.7 (s, 3H, C(11)CH_3), 2.5 (q, J= 7.5 Hz, 2H, $CH_3\underline{CH_2}$), 2.0-2.7 (m, 8H, CH_2C=), 5.12 (bs, 2H, CH=C). Tlc analysis showed a single spot (Rf 0.31; 5% ethyl acetate in hexane). Glc analysis showed one peak (tr 20.17; system A, 120°). The CIMS showed a base peak at m/e 209 corresponding to the molecular ion ($C_{14}H_{24}O + H^+$). Salient peaks were observed at m/e 191 (MH^+-18) and 153 (MH^+-56).

Ethyl-3-Ethyl-7,11-dimethyl-2(E),6(E),10-dodecatrienoate (40). Reaction of ketone 39 (17.58 g, 85 mmol) with the sodium salt from 21 g (93 mmol) of triethyl phosphonoacetate, as described in the preparation of ethyl farnesoate, gave 19 g (86%) of a 3:2 mixture of the 2E and 2Z isomers of 40. Spinning band distillation, maintaining a reflux ratio of 15:1 and a reflux rate of about 16 drops/min, gave the following fractions:

Fraction	Weight (g)	% all-(E)	Fraction	Weight (g)	% all-(E)
1	0.916	14	9	0.895	48
2	0.453	17	10	0.887	52
3	0.447	20	11	1.205	60
4	0.584	32	12	0.802	85
5	1.020	24	13	0.600	79
6	1.460	32	14	1.246	85
7	1.072	29	15	0.760	94
8	0.750	33	Residue	4.774	98

Filtration of the remaining pot residue through basic alumina (grade II, 30 g, pet-ether solvent), concentration *in vacuo* and bulb-to-bulb distillation (96°/0.05 mm) yielded 3.5 g of the desired compound (2E/2Z = 98/2) ir 5.80 (s, C=O), 6.13 (s, C=C), 6.78 (s), 6.87 (s), 7.30 (s), 7.87 (m), 8.33 (vs), 8.77 (vs), 9.13 (w), 9.64 (s),

and 11.56 (m); nmr (No. 9) 1.05 (t, J= 7.5 Hz, 3H, $\underline{CH_3}CH_2$), 1.26 (t, J= 7.0 Hz, 3H, $\underline{CH_3}CH_2O$), 1.6 (s, 6H, C(7,11)CH_3), 1.68 (s, 3H, C(11)CH_3), 1.93-2.33 (m, 8H, CH_2C=), 2.65 (q, J=7.5 Hz, 2H, $CH_3\underline{CH_2}$), 4.17 (q, J=7.0 Hz, 2H, $CH_3\underline{CH_2}O$), 5.15 (bs, 2H, C(6,9)H), and 5.67 (s, 1H, C(2)H). Tlc analysis showed one spot (Rf 0.40, 5% ethyl acetate in hexane). Glc analysis showed two peaks in the ratio mentioned above (system A, 150°; 2 Z isomer 18.8 min, 2 E isomer 20.8 min). The CIMS spectrum showed a base peak at m/e 279 (MH^+). Salient peaks were observed at m/e 233 ($MH^+-C_2H_5OH$), and 205 (MH^+-74).

Anal. Calcd for $C_{18}H_{30}O_2$: C, 77.65%; H, 10.86%. Found: C, 77.62%; H, 10.82%.

3-Ethyl-7,11-dimethyl-2(E),6(E),10-dodecatrien-1-ol (38). A stirred solution of ester 40 above (627 mg, 2.25 mmol) in 5 ml of dry ether was treated with sodium bis-(2-methoxyethoxy) aluminum hydride (Realco Vitride, 0.691 ml of a 3.58 M solution in benzene, 2.47 mmol) under nitrogen at 0°. After stirring for 3 hr at 25°, water was added until a heavy precipitate formed. To this mixture was added about 1 g of anhydrous magnesium sulfate and the stirring was continued for 15 min. Suction filtration using a sintered funnel, concentration in vacuo, and bulb-to-bulb distillation (98°/0.075 mm) yielded 501 mg (94%) of pure alcohol 38: ir 3.02 (vs, OH), 5.97 (w, C=C), 6.83 (w), 7.22 (w), and

10.00 (s); nmr 0.99 (t, J=7.5 Hz, 3H, $\underline{CH_3}CH_2$), 1.58 (s, 6H, C(7,11)CH_3), 1.69 (s, 3H, C(11)CH_3), 1.83-2.35 (m, 10H, CH_2C=), 2.39 (s, 1H, exchanges with D_2O, OH), 4.15 (d, J=7 Hz, 2H, CH_2O), 5.15 (bs, 2H, C(6,10)H), and 5.40 (t, J=7 Hz, 1H, C(2)H). Tlc analysis showed a single spot (Rf 0.35; 10:1 benzene-ehtyl acetate). Glc analysis showed two peaks in the ratio 2Z:2E, 2:98[93] (system A, 150°; 2Z, 18.75 min 2E , 20.36 min). The CIMS showed a base peak at m/e 219 (MH^+-H_2O). Salient peaks were observed at m/e 205, 163, and 149.

Anal. Calcd for $C_{16}H_{28}O$: C, 81.29%; H, 11.94%. Found: C, 81.29%; H, 11.86%.

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(93) A series of nmr spectra was taken for each isomer with increasing amounts of the shift reagent Eu(fod)$_3$[36]. The effect on the 3-ethyl protons is given below:

Compound with higher tr (glc) and lower Rf (tlc). Concentration 101.4 mg/300µl $CDCl_3$.

Eu(fod)$_3$ (mg)	Ratio	$\underline{CH_3}CH_2$	$CH_3\underline{CH_2}$	C(4)H
0.0	0.0000	0.98	2.11	2.09
13.3	0.1312	1.12	2.31	2.20
23.7	0.2337	1.22	2.48	2.29
38.1	0.3757	1.35	2.72	2.40
50.0	0.4931	1.46	2.86	2.50
70.0	0.6903	1.66	3.23	2.71
93.0	0.9172	1.84	3.47	2.87

Compound with lower tr (glc) and higher Rf (tlc). Concentration 64.11 mg/300µl $CDCl_3$.

Eu(fod)$_3$ (mg)	Ratio	$\underline{CH_3}CH_2$	$CH_3\underline{CH_2}$	C(4)H
0.0	0.0000	1.02	2.07	2.08
7.0	0.1092	1.06		2.18
13.5	0.2106	1.14		2.31
20.8	0.3244	1.23	2.33	2.48
26.6	0.4149	1.30	2.42	2.72
43.1	0.6723	1.49	2.63	3.12
51.9	0.8095	1.62	2.77	3.41

By plotting the shifts of the 3-ethyl protons versus the ratio shift reagent/substrate the graph in Fig. 17 is obtained.

The isomer in which the 3-ethyl was more responsive to the shift reagent (eg. higher tr, lower Rf) was assigned 2E stereochemistry.

5,9-Dimethyl-4(E),8-decadien-1-al (42). A stirred suspension of pyridinium chlorochromate (4 g, 18.6 mmol) and anhydrous sodium acetate (305 mg, 3.71 mmol) in 16 ml of CH_2Cl_2 was treated with a solution of 5,9-dimethyl-4(E),8-decadien-1-ol (41)[29b] (2.26 g, 12.4 mmol) in anhydrous CH_2Cl_2 at room temperature. There was a slight increase in the temperature of the reaction and formation of a black precipitate during the course of the 2.5 hr oxidation.[44] The reaction was treated with 80 ml of ether and the residual solid washed with ether several times. The combined ether extracts were concentrated in vacuo to give a dark yellow oil. Addition of hexane (20 ml) precipitated a brown solid. Suction filtration using a sintered funnel with a celite pad and concentration in vacuo left 1.972 g of crude reaction mixture which was chromatographed on silica gel (30 g, 24 x 2 cm column). The results of this separation are given below.

Fraction	Solvent	Volume (ml)	Weight (g)	Rf of Components (10% ethyl acetate in hexane).
1	Hexane	200		
2	Hexane-ethyl acetate (99:1)	100		
3	" " " (98:2)	"		
4	" " " (95:5)	"	0.516	0.61, 0.46
5	" " " "	"	0.756	0.46
6	" " " "	"	0.050	0.46, 0.34, 0.29, 0.23

Fraction 4 was bulb-to-bulb distilled to give pure aldehyde, which was combined with fraction 5 to give after a second bulb-to-bulb distillation 1.1 g (49%) of pure 42: ir 3.68 (m, -CO-H) and 5.75 (s, C=O); nmr 1.62 (s, 6H, C(5, 9)CH_3), 1.68 (s, 3H, C(9)CH_3), 1.83-2.60 (m, 8H, CH_2C=), 5.08 (bs, 2H, vinyl H) and 9.7 (m, 1H, C(1)H). Glc analysis (System A, 110°) showed a major component (94%, tr 13.13min), contaminated with a minor impurity (6%, tr 11.89 min).

3-Desmethylnerolidol (43). Into a dry 50 ml three-necked, round-bottom flask, provided with a magnetic stirrer, nitrogen lines, and a rubber septum on one side neck was placed 1 g (4.4 mmol) of tetravinyl tin[45] by means of a syringe. n-Buthyllithium (13.2 mmol, 6 ml of a 2.2 M solution, was added dropwise and a white precipitate formed.[45] After stirring 30 min at 0°, the solvent was removed under a stream of nitrogen. The residual solid was washed with petroleum ether, and the solvent removed by suction with a gas dispersion tube. The remaining white solid was dissolved in 10 ml of ether and treated with a solution of aldehyde 41 (0.89 g, 4.9 mmol) in 3 ml of ether at 15°. No starting material could be detected by tlc after stirring for 30 min at room temperature. The mixture was treated with brine (5 ml), the layers separated, and the aqueous phase extracted with ether (2 x 10 ml). The combined organic extracts were washed with water, dried and concentrated in vacuo to give

1.3 g of crude reaction mixture. Tlc analysis (benzene-ethyl acetate, 10:1) showed one major product (43), (Rf 0.48) and 6 minor impurities (Rf 0.1, 0.25, 0.29, 0.37, 0.61, and 0.92). This mixture was chromatographed on silica gel (30 g, 23 x 2 cm column) using hexane (200 ml) and 1% ethyl acetate in hexane (300 ml) as eluting solvents. The fractions containing compound 43 were combined to give 806 mg (79%) of a colorless mobile oil: ir 3.00 (vs, OH), 5.39 (w, terminal vinyl), 5.97 and 6.02 (w, C=C). Nmr (No.10) 1.5 (m, 2H, C(4)H), 1.63 (m, 6H, C(7,11)CH_3), 1.70 (s, 3H, C(11)CH_3), 1.82-2.38 (m, 9H, CH_2C=, OH), 4.11 (m, 1H, C(3)H), 4.97-5.42 (m, 4H, vinyl H), and 5.62-6.23 (m, 1H, vinyl H) Glc analysis (System A, 150°) showed a major component (94%, tr 5.63 min) contaminated with a minor impurity (6%, tr 5.06 min).

7,11-Dimethyldodeca-2(E),6(E),10-trien-1-al (46). Activated manganese dioxide[89] (15 g, 173 mmol) was added in portions over a period of 15 min to a stirred solution of 7,11-dimethyldodeca-2(E),6(E),10-trien-1-ol (31)[23] (2 g, 9.62 mmol) in 80 ml hexane at 0°. The mixture was stirred for 30 min after which no starting alcohol could be detected by tlc. Suction filtration using a sintered funnel, removal of solvents in vacuo, and bulb-to-bulb distillation (70°/0.075 mm) gave pure aldehyde 46 as a colorless liquid (1.332 g, 67%): ir (No.1) 3.55, 3.67 (m, -COH), 5.87 (s, C=O), 6.07 (w, C=C), 6.88 (m), 7.19 (w), 8.60 (m), 8.90 (s), 9.91 (w), and 10.28 (s); nmr[51] No. 11) 1.60 and 1.67 (s, 9H,

$CH_3C=$), 1.90-2.50 (m, 8H, CH_2), 4.80-5.33 (bs, 2H, C(6,10)H), 5.94 (d of d, J=7.5 Hz, and 15.5 Hz, 1H, C(2)H), 6.83 (d of t, J=6 and 15.5 Hz, 1H, C(3)H), 9.45 (d, J=7.5 Hz, 1H, C(1)H). Tlc analysis showed one spot (Rf 0.38; 5% ethyl acetate in hexane). Glc analysis showed one peak (tr 6.95 min, System A, 160°).

1-H^3-7,11-Dimethyl-2(E),6(E),10-dodecatrien-1-ol (31). The title compound was prepared from 0.485 mmol of aldehyde 46, and the sequence lithium aluminum hydride (0.037 mmol), tritium labeled lithium aluminum hydride (0.037 mmol, sp. act. 135.35 mC/mmol), and lithium aluminum hydride (0.0527 mmol) as described for the preparation of labeled farnesol. The product, obtained in 95% yield, has the same Rf (0.29; 10:1, benzene-ethyl acetate) on tlc as the authentic unlabeled material.

1-H^3-2,3,7,11-Tetramethyl-2(E),6(E),10-dodecatrien-1-ol (36). This radioactive compound was prepared from 0.3772 mmol of ethyl ester 37 using the sequence of hydrides (0.0792 mmol; 0.0369 mmol with sp. act. 132 mC/mmol,and 0.0792 mmol) as previously described for the preparation of labeled farnesol. The product (87 mg, 98% yield) was found to cochromatograph on tlc (Rf 0.29; 10:1 benzene-ethyl acetate) with a sample of the authentic unlabeled material.

1-H^3-7,11-Dimethyl-3-ethyl-2,6,10-dodecatrien-1-ol (38). This compound was prepared from 0.5 mmol of ethyl ester 40 and the sequence lithium aluminum hydride (0.1926 mmol), tritium labeled lithium aluminum hydride (0.0377 mmol, sp. act. 135.36 mC/mmol), and lithium aluminum hydride (0.0449 mmol) as described for the preparation of labeled farnesol. The product obtained in 95% yield has the same Rf (0.35; 10:1, benzene-ethyl acetate) on tlc as a sample of authentic unlabeled material.

2,3,7,11-Tetramethyldodeca-2(E),6(E),10-trien-1-yl-bromide (51). A solution of phosphorus tribromide (180 mg, 0.665 mmol) in 0.4 ml of dry ether was added dropwise with the exclusion of light to 0.522 g (1.75 mmol) of alcohol 36 (2Z:2E, 2:98) in 2ml of dry ether at 0. After stirring under nitrogen for 90 min, the mixture was poured into 10 g of ice-water and extracted twice with 20 ml ether. The combined extracts were washed with water, dried over sodium sulfate-magnesium sulfate, and concentrated in vacuo to furnish 0.50 g (76%) of a light yellow oil: ir 6.02 (m, C=C), 6.87 (s), 7.21 (m), and 8.33 (s); nmr 1.60 (s, 6H, C(7,11) CH_3), 1.75 (s, 6H, C(2,3)CH_3), 1.9-2.2 (m, 8H, CH_2), 4.03 (s, 2H, C(1)H), and 5.15 (bs, 2H, vinyl). Tlc analysis showed one spot (Rf 0.73; hexane).

11,14-Dimethylsqualene (47). A solution of 2-methyl farnesyl bromide (51) (1.45 g, 4.86 mmol; 2Z:2E,14:86) in 50 ml of dry DMF was placed into a 250 ml three-necked flask provided with magnetic stirrer, reflux condenser (gas outlet), flow adapter (nitrogen inlet), and a rubber septum. The system was alternately evacuated to aspirator pressure and filled with nitrogen four times. A positive nitrogen pressure was maintained throughout the course of the reaction, the gas coming out of the reaction bubbling through concentrated nitric acid to destroy any nickel tetracarbonyl carried over.[94] Nickel tetracarbonyl (2.8 ml, 21.6 mmol) was added by syringe and the mixture stirred for 17 hr at 42°. Excess nickel tetracarbonyl was codistilled with 50 ml ether, the residue poured into 50 ml of cold 5% hydrochloric acid, and the mixture extracted with pet-ether (3 x 50 ml). The extracts were washed with 5% sodium bicarbonate (2 x 50 ml), water (2 x 50 ml), and finally dried over sodium sulfate-magnesium sulfate to yield, after concentration in vacuo, 1.102 g of a light yellow oil. Tlc analysis showed three spots (Rf 0.00, 0.33, and 0.55; hexane). The mixture was chromatographed on silica gel (50 g, 2 x 38 cm column) using petroleum ether. The fractions with Rf 0.33 were mixed to give 604 mg (57%) of a colorless liquid. Glc analysis (System A, 235°) of the purified product revealed the

(94) This reaction was done in a well ventilated hood since nickel tetracarbonyl is a volatile, highly toxic compound. For a review about the handling and techniques suitable for this versatile reagent see ref. 53 and 54.

presence of five components (Fig. 37C) in the ratio and with the retention times indicated below.

Component	Fig. 37C Peak No.	Retention time (min)	% of total mixture
10Z, 14 E	1	6.69	21
10Z, 14 E	3	8.19	34
10E, 14 E	5	10.42	16
Unidentified	2	6.94	16
Unidentified	4	9.11	13

Stereochemical assignment to these isomers is discussed in chapter 2, section B. A sample of the clathrate purified (E),(E)-isomer has the following properties: ir 5.95 (w, C=C), 6.01 (w, C=C), 6.83 (s), 7.22 (m), 8.70 (w), 9.04 (w), 11.27 (w), and 11.83 (w); nmr (No. 12) 1.63 (s, 12H, C(2,6,19,23)Z CH_3), 1.67(s, 6H, C(2,23)E CH_3), 1.68 (s, 2H, C(10,11,14,15) CH_3), 1.81-2.33 (m, 20 H, CH_2), 4.83-5.42 (bs, 4H, vinyl H). A sample of pure compound 47 was obtained by preparative glc (System E, 240°, tr 13 min). The chemical ionization mass spectrum of this sample is described in chapter 2, section B.

Catalytic Hydrogenation. A fraction of the purified mixture from the reaction above (240 mg, 0.547 mmol) was hydrogenated in the presence of 10% Pd/charcoal catalyst (about 5%) as described for the preparation of hexahydro ethyl farnesoate (35). After work up, 235 mg (95%) of clear liquid were obtained. The ir spectrum did not show double bond absorptions; nmr 0.86 (d, J=5.5 Hz, 30 H, CH_3), 1.02-1.78 (m, 36H, CH_2, CH). Glc analysis (System A, 215°) showed five peaks (Fig 37F) with the retention times and in the ratio shown below.

Component	Retention time (min)	% in mixture
1	7.39	2
2	8.06	11
3	9.15	20
4	10.13	65
5	12.19	2

Purification by thiourea clathrate. Thiourea was dissolved in methanol (150 ml) at the reflux point until an excess of salt did not dissolve. The solution was allowed to crystalize for at least 8 hr at room temperature.

The purified nickel carbonyl reaction mixture (256 mg) was suspended in 25.6 ml of the supernatant solution prepared above. The mixture, contained in a centrifuge tube, was shaken for 14 hr at 25°, after which no crystals were formed.

Crystallization was achieved by adding a small crystal of thiourea and stirring 14 hr as before. The crystals were collected by centrifugation, washed twice with pet ether, dissolved in 2 ml of water, and the product obtained extracted with pet-ether. The extracts were dried, filtered, and concentrated *in vacuo* to give 36 mg of a clear oil. Glc analysis (System A, 235°) showed four components (Fig. 37G) given below.

Component	Retention time	% of total mixture
10Z, 14Z	6.56	3
10Z, 14Z	8.06	6
10E, 14E	10.28	89
Unidentified	9.19	2

Note. This reaction was carried out as described earlier starting with a mixture of 2-methylfarnesyl bromide (2Z:2E , 69:31; 0.746 g, 2.5 mmol) and nickel tetracarbonyl (1.5 ml, 1.98 g, 11.6 mmol). After 3 hr at 25°, the mixture was warmed for 20 hr at 50°. Work up as before yielded 509 mg of crude mixture. Separation as previously described using 7.5 g of silica gel gave 132 mg (24%) of a clear liquid. Glc analysis (System A, 235°) showed four of the peaks present in the previous reaction mixture. Their relative retention times and percentages are presented in the following table.

Component	Fig. 37B Peak No.	Retention Time (min)	Relative percentages
10Z, 14 Z	1	6.49	38
10Z, 14 Z	3	8.03	41
10E, 14 Z	5	10.13	14
Unidentified	4	8.89	7

11,14-Dimethylsqualene (47). Into a stirred solution of pyrrolidine (485 mg, 6.76 mmol) in 5 ml of dry ether was injected 2.94 ml of n-butyllithium in hexane (2.3 M solution, 6.76 mmol) at 0°. A white precipitate formed in 10 min; and 1.286 g of cuprous iodide (3.38 mmol) was added at once. The black mixture formed was stirred for 30 min at 0°, and treated with a solution of E,E-2-methylfarnesyl bromide (51) (2Z:2E , 2:98; 0.5 g, 1.69 mmol) in 5 ml of ether. A dark yellow precipitate formed and the stirring was continued for 4 hr at 0° after which the reaction was stopped by careful addition of dilute hydrochloric acid and pet-ether. Filtration using a sintered funnel, extraction with pet-ether, drying and concentration in vacuo left 331 mg of crude product as a dark yellow oil. Tlc analysis showed three spots (Rf 0.00, 0.33 and 0.55; hexane). The component with Rf 0.33 was separated in 70% yield by preparative tlc using silica gel plates in hexane. Glc analysis (System A, 235°) of the purified product showed five components

(Fig. 37A) tabulated below.

Component	Fig. 37A Peak No.	Retention time (min)	% Total mixture
10Z , 14Z	1	6.64	10
10Z , 14E	3	8.19	28
10E , 14E	5	10.44	31
Unidentified	2	6.90	10
Unidentified	4	9.11	21

Stereochemical assignment of these isomers is discussed in chapter 2, section B.

A sample of the clathrate purified isomer has the same physical properties and spectra as the sample prepared from the nickel tetracarbonyl reaction.

Hydrogenation. The purified mixture from the reaction above (55 mg, 0.125 mmol) was completely hydrogenated in the presence of 10% palladium-on-charcoal catalyst (about 2.5 mg) following the procedure described for the preparation of hexahydro ethyl farnesoate(35), yielding, after work up, 50 mg (89%) of a clear liquid. Glc analysis (System A, 215°) showed three components (Fig. 37D) with the retention times shown in the following table.

Component	Fig. 37D peak No.	Retention time (min)	% of total mixture
1	6	8.63	9
2	7	9.90	67
3	8	12.19	24

The ir and nmr spectra of this mixture were similar to those obtained from hydrogenation of the purified nickel tetracarbonyl reaction.

Purification by thiourea clathrate. The purified mixture from the reaction above (168 mg) was mixed with 16.8 ml of saturated thiourea solution and subjected to crystallization as described for the purification of the sample prepared from the nickel tetracarbonyl reaction. After work up, 17 mg of purified product were obtained. Glc analysis (System A, 235°) showed the components in the ratio given below.

Component	Retention time (min)	% of total mixture
10Z, 14Z	6.64	1
10Z, 14E	8.19	9
10E, 14E	10.44	86
Unidentified	9.11	4

Identification of the Hydrocarbon Product from Incubation of 1-^{3}H-2-Methylfarnesyl Pyrophosphate and an Extract of Squalene Synthetase from Yeast.

Into a 500 ml erlenmeyer flask was placed the following incubation mixture in a total of 97.8 ml: potassium phosphate buffer (pH 7.5), 50 mM; water, 75 ml; $MgCl_2$, 10 mM; NADPH, 1.6 mM; [1-^{3}H]-2-methylfarnesyl pyrophosphate 2.18 μM; farnesyl pyrophosphate, 1 μM. The mixture was flushed with nitrogen for 5 min, prewarmed at 37°, and treated with an extract of squalene synthetase (9.38 mg of protein). After 90 min in a reciprocating bath at 37° the incubation mixture was diluted with 196 ml of absolute ethanol and extracted with distilled hexane (3 x 300 ml). The combined extracts were concentrated in vacuo. A small aliquot of this residue was spotted on two silica gel plates (2.5 x 10 cm, Eastman Kodak) and separated in hexane. One plate was developed with iodine vapors, revealing two spots (Rf 0.06-0.38 and 0.69-0.78). The two plates were cut equally in 5 sections, placed in vials with scintillation liquid, and assayed for radioactivity. The results are tabulated below.

Section	Rf	cpm: with I_2	without I_2
1	0.00 - 0.06	71	76
2	0.06 - 0.38	141	136
3	0.38 - 0.69	29	25
4	0.69 - 0.78	366	295
5	0.78 - 1.00	24	20

The residue was also subjected to glc analysis (System A, 220°) showing 3 major peaks (Fig. 38A), one coincident with squalene (tr 11.63 min), and two unresolved peaks (tr 15.38 min and 15.75 min).

Part of the residue was chromatographed on silica gel plates (Analtech) prewashed twice with chloroform. Together with the preparative plates was run an analytical plate of the residue and squalene. After five developments in hexane the analytical plate showed one spot at the origin and another one with the same Rf as squalene (0.69). The two bands corresponding to these two spots were scraped from the preparative plate, each eluted with 4 ml of chloroform, and the extracts concentrated under a stream of nitrogen in conical tubes. The fraction at the origin showed one major component using both the same GC system as above (tr 16.69 min), and system C (tr 8.63 min). This component was detected in most of the incubations carried out in these laboratories. An aliquot of the second tlc band (Rf 0.70) was subjected to preparative glc (System D, 200°) using a splitter column with approximately 80% collecting efficiency. Dry-ice acetone cooled collection tubes (2 x 300 mm) were employed and the collected product was eluted into scintillation vials using 2 ml of hexane. The solutions were diluted with 15 ml of scintillation fluid and finally assayed for radioactivity (Fig. 38C). The results of these determinations are given in the following table.

Retention time of fraction (in min)	Radioactivity (cpm)	% Recovered[a]
0.00 - 11.25	84	17
11.25 - 13.88[b]	122	25
13.88 - 15.00	43	9
15.00 - 18.75[c]	114	23
18.75 - 56.25	34	7

[a]An equal volume of the aliquot injected (1.5 µl) was assayed directly for radioactivity to give 497 cpm.
[b]Squalene had a retention time of 12.45 min under identical conditions.
[c]11-methylsqualene had a retention time of 16.88 min.

Glc analysis showed one peak with the same retention time as squalene (System A, 220°, 12.38 min; System C, 14.8 min), and another one coincident with 11-methylsqualene (System A, 220°, 16.20 min; System C, 19.35 min) while no peak was detected where 11,14-dimethylsqualene appears (System A, 220°, 23.25 min; System C, 24.11 min). A second aliquot was subjected to Glc-mass spectrometry[58] (System C); the spectrum obtained (Fig. 39B) is discussed in chapter 2, section B.

Identification of the Hydrocarbon Product from Incubation of 1-^{3}H-3-Desmethylfarnesyl Pyrophosphate and an Extract of Squalene Synthetase from Yeast.

The scale of the normal assay incubation[48a] containing [1-^{3}H]-16 was increased 100 fold. The procedure for this incubation was similar to that described above, except that the time of incubation was 3 hr. The incubation mixture was terminated with 200 ml of ethanol and 100 ml of 8 N potassium hydroxide, and was then heated for 1 hr at 70°. The hydrocarbon product was extracted with hexane (3 x 300 ml), the extracts concentrated *in vacuo*, and the residue chromatographed on neutral alumina (0.6 x 5 cm column) using 5% ether in hexane as eluting solvent. The combined fractions were concentrated and an aliquot assayed by preparative gc-liquid scintillation counting (System D, 190°). The results are given in the following table.

Retention time of fraction (in min)	Radioactivity (cpm)
0.00 - 15.00[a]	22
15.00 - 16.13	19
16.13 - 17.25	193
17.25 - 18.00[b]	456
18.00 - 18.75	214
18.75 - 19.50	47
19.50 - 22.88[c]	23
22.88 - 60.00	64

[a]Didesmethylsqualene (49) had a retention time of 14.25 min (Fig. 40).

[b]Monodesmethylsqualene (50) had a retention time of 18 min.

[c]Squalene had a retention time of 25.88 min.

A second aliquot was subjected to glc-mass spectrometry[58]. The spectrum obtained (Fig. 41A) is discussed in chapter 2, section B.

4,8,12-Trimethyl-1,3(E),7(E),11-tridecatetraene (63).

Into a solution of methyltriphenylphosphonium bromide (5.455 g, 15.27 mmol) in 87 ml dry THF were injected dropwise 6 ml of n-butyllithium in hexane (2.4 M solution, 14.40 mmol) at 0°. The orange ylide solution formed was stirred at ambient temperature for 30 min, then cooled at -78° and treated with a solution of farnesal (2Z:2E, 3:97; 2.188 g, 9.93 mmol) in 5 ml of dry THF. The resulting mixture was stirred between -30 and -25° for 30 min, 0° for 30 min, and 25° 30 min. The hydrocarbon formed was separated from triphenylphosphine oxide by careful addition of water (0.26 ml, 14.4mmol), hexane (100 ml), and anhydrous $MgSO_4$ (10 g), followed by suction filtration using a sintered funnel. The residue was washed with hexane several times. The combined extracts were concentrated in vacuo, filtered through silica gel (15 g, 2 cm i.d. column) with 150 ml of hexane, and bulb-to-bulb distilled (88°, 0.075 mm) to give 2.045 g (94% yield) of pure tetraene 63 as a colorless mobile liquid: ir (No.2) 3.25 and 3.58 (w, term. vinyl), 6.01 and 6.20 (w, diene), 6.90 (m), 7.00 (w), 7.20 (w), 10.15 (s), and 11.17 μ (s); nmr (No. 14) 1.62, 1.68, and 1.77 (3s, 12H, allyl CH_3), 1.92-2.32 (m, 8H, allyl CH_2), 4.83-5.34 (m, 4H, vinyl), 5.68-6.01 (m, 1H, vinyl), and 6.28-6.93 ppm (m, 1H, vinyl). Tlc analysis showed one spot in hexane (Rf 0.54). Glc analysis showed 2 peaks (2Z:2E, 3:97; tr 8.14, 2Z; 8.81, 2 E ; System A, 125°). CIMS m/e 219 (MH^+).

Anal. Calcd for $C_{16}H_{26}$: C, 88.00%; H, 12.00%. Found: C, 86.46%; H, 11.67%.

N.N-Dimethylisobutyramide (59a).[75] A 200 ml 2-necked round-bottom flask provided with a dry-ice condenser was charged with a solution of isobutyryl chloride[95] (25.6 g, 0.24 mol) in 25 ml of dry benzene. The solution was treated with excess anhydrous dimethylamine for 3 hr at 0°. The product formed was isolated by suction filtration using a sintered funnel and benzene washings of the residue. The combined benzene extracts were concentrated in vacuo affording a yellow crude product. Distillation through a short Vigreux column yielded 20.2 g (73%) of pure amide 59a: bp 176-178°[75a]; ir 6.06 (s, C=O); nmr[75b] 1.09 (d, J=6.5Hz, 6H, C(2)CH_3), 2.85(m,1H, C(2)H), 2.93 and 3.09 (s, 6H, N-CH_3).

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(95) Isobutyryl chloride was purchased from Aldrich and distilled before use.

(96) This amine in turn was generated by stepwise addition of NaOH 5 N (200 ml) over N,N-dimethylamine hydrochloride 5 N (200 ml) at 50-85°. The generated amine was passed through a $CaCl_2$ tube and bubbled into the acid chloride solution.

N,N-Dimethyl-1-chloro-2-methylpropenylamine (61a).

A 100 ml four-neck round-bottom flask was equipped with a magnetic stirrer, two flow adapters, a rubber septum, and a gas dispersion tube. A teflon tubing fitted with hypodermic needles at each end was inserted at one extreme through a rubber septum into the gas dispersion tube, while the other end of this tubing was closed to the atmosphere with a rubber septum. The system was charged with a solution of N,N-dimethylisobutyramide (3.15 g, 27.35 mmol) in 50 ml of dry benzene. A positive nitrogen pressure was maintained throughout the course of the reaction except during phosgene addition, at which time outgoing phosgene was bubbled through a concentrated sodium hydroxide solution. Phosgene (62.5 g, 625.4 mmol) was slowly bubbled in for 1 hr at 0°, and 2 hr at ambient temperature. White crystals formed and the reaction was stirred for 18 hr at 25°. The gas exit line was connected to a $CaCl_2$ tube and excess phosgene removed by water aspirator vacuum for 2 hr. The residual mixture was washed with pet-ether (3 x 30 ml), the washings being removed by positive-pressure-filtration through the gas dispersion tube. Concentration of the washings afforded 650 mg of starting amide, 2.5 g (21.70 mmol) having been consumed. The light yellow residue in the reaction flask was dissolved in 40 ml of CH_2Cl_2 and treated with 3.1 ml of triethylamine (2.25 g, 22.28 mmol) under a fast nitrogen flow to prevent back pressure into the nitrogen line due to

a slight increase in temperature. Triethylamine hydrochloride was completely precipitated after stirring for 15 min by adding 20 ml of pet-ether and removed by positive pressure filtration. The filtrate and washings of the residue (2 x 20 ml of pet-ether) were directly injected into a second flask and concentrated with water aspirator vacuum at room temperature to give 2.686 g (54% yield by nmr assay) of a dark yellow liquid: nmr analysis (No. 17) showed two singlets of equal intensity at 1.77 and 2.38 δ^{83} in about 58%. Contaminants observed were starting amide, benzene, dichloromethane, and pet-ether. This mixture was used without any further purification.

3-(2,6,10-Trimethylundeca-1(E),5(E),9-trienyl)-2,2-dimethylcyclobutanone (53a). A solution of 4,8,12-trimethyl-1,3(E),7(E),11-tridecatetraene (63) (652 mg, 2.99 mmol) in 15 ml of dry CH_2Cl_2 was injected into a suspension of $AgBF_4$ (6.28 mg, 3.23 mmol) in 15 ml of CH_2Cl_2 at room temperature. The solution formed was cooled to -78° and treated with 691 mg of the crude α-chloroenamine prepared above (3 mmol). A white precipitate formed as the reaction was slowly allowed to reach room temperature at which time it was stirred for 1 hr. Filtration and concentration in vacuo left a dark oil which was treated with 10 ml of 1 N sodium hydroxide and extracted with pet-ether (3 x 5 ml). The combined extracts were washed with water (3 x 5 ml), dried, filtered, and concentrated in vacuo to give 815 mg of a yellow oil. The crude

product was dissolved in 1 ml of hexane and chromatographed on silica gel (8g, 1 x 20 cm column). The column was eluted first with pet-ether (50 ml), 1% ethyl acetate in hexane (50 ml), and finally with 2% ethyl acetate in hexane (70 ml). The fractions containing a single spot on tlc (Rf 0.27 in 3% thyl acetate in hexane) were mixed and bulb-to-bulb distilled ($102^{o}/0.025$ mm) to give 507mg (59%) of pure cyclobutanone 53a as a colorless mobile liquid: ir (No. 4) 5.59 (s, C=O in 4-member ring), 5.97 (w, C=C), 7.97 (w), 8.64 (mw), and 9.44 μ (m); nmr (no.18) 1.03 and 1.22 (s, 6H, cyclobutyl methyls), 1.63 and 1.68 (s, 12H, CH_3C=), 1.88-2.23 (m, 8H, CH_2C=), 2.68-3.35 (m, 3H, cyclobutyl H), and 4.87-5.33 ppm (m, 3H, vinyl H). Glc analysis showed a single peak (tr 13.99 min; System A, 175^{o}). The CIMS showed a molecular ion at m/e 289 ($M+H^+$) and prominent peaks at 271, 261, 246, 223, and 203.

Anal. Calcd for $C_{20}H_{32}O$: C, 83.27%; H, 11.18%. Found: C, 82.98%; H, 11.05%.

3-(2,6,10-Trimethylundeca-1(E),5(E),9-trienyl)-2,2-dimethylcyclobutanol(54). A solution of dimethylcyclobutanone 53a (200 mg, 0.69 mmol) in 2 ml of ethanol was added to 6ml of a 0.1M sodium borohydride solution in ethanol (6 mmol) at ambient temperature. After 1 hr stirring the solvent was removed in vacuo and the residue treated with 3 ml of brine and extracted with ether (3 x 5 ml). The ether extracts were dried and concentrated to give 0.1966 g of a colorless mobile liquid. Tlc analysis showed two spots (Rf 0.51 and 0.54; 10:1, benzene-ethyl acetate). Glc analysis showed two peaks in the ratio 3:97 (tr 13.13 and 21.56 min, System A, 175°). The small amount of impurity was removed by preparative tlc[94] (10:1, benzene-ethyl acetate as solvent) affording 192 mg (95% yield) of pure dimethylcyclobutanol 54[97] (compound with Rf 0.51 and tr 21.56 min): ir 3.00 (vs, OH), 5.76 (w, C=C), 5.93 (w, C=C), 6.78 (m), 7.14 (w), 8.03 (w), 8.23 (w), 8.66 (w), 8.85 (w), 9.09 (m, C-O), 9.39 (w), 9.78 (m), and 10.20 μ(w); nmr (No. 19) 0.93 and 1.07 (2s, 6H, cyclobuthyl CH_3), 1.62 and 1.69 (2s, 12H, CH_3C=), 1.84-2.29 (m, 8H, CH_2C=), 2.16 (s, 1H, OH), 2.33 (m, 3H, cyclobutyl H), 3.8 (m, 1H, C(1)H), and 4.92-5.34 ppm (m, 3H, vinyl H). The CIMS showed a molecular ion peak at m/e 291 ($M+H^+$) and prominent peaks at m/e 273 (base peak), 245, 231, 219, 203, 191, 137, 123 and 119.

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(97) Partial decomposition is observed when this compound is bulb-to-bulb distilled.

Anal. Calcd for $C_{20}H_{34}O$: C, 82.69%; H, 11.80% Found: C, 82.13%; H, 11.64%.

The nmr shifts of the cyclobutyl methyls at different concentrations of $Eu(fod)_3$ are given as follow:

$Eu(fod)_3$ added (mg)	ratio[a]	Shift of cyclobutyl methyls (ppm)
0.0	0.0000	0.92 and 1.07
7.0	0.1512	1.30 and 1.37
13.3	0.2873	1.54 and 1.86
24.1	0.5205	1.84 and 2.45
33.0	0.7127	2.12 and 2.99
48.6	1.0497	2.57 and 3.90

[a]The concentration of cyclobutanol 54 was 46.3 mg dissolved in 150 µl of $CDCl_3$.

The plotting of the shifts of the methyl signals versus the ratio of shift reagent to compound is shown in Fig. 49.

1,5,9-Trimethyl-4(E),8-decadien-1-ol (65). A solution of E-geranylacetone[29] (40.25 g, 0.2075 mmol) in 180 ml of dry ethanol was added dropwise to a suspension of sodium borohydride (3.7 g, 0.098 mmol) in 100 ml of absolute ethanol over a period of 30 min at 25°. There was a slight increase in the temperature of the reaction during the addition. After stirring the mixture for 1 hr, concentration in vacuo left a light yellow residue that was treated with 200 ml of saturated ammonium chloride solution, which was extracted with ether (3 x 150 ml). The combined ether extracts were washed with 100 ml of saturated ammonium chloride solution, dried, and concentrated in vacuo to furnish 40.5 g (100 % yield) of alcohol 65 essentially pure: ir 2.99 (vs, OH), 5.99 (w, C=C), 6.85 (m), 7.22 (m), 8.89 (m), 9.04 (sh), 9.26 (m), 10.20 (w), 10.50 (w), 10.73 (w), 11.03 (w), and 12.05 (m); nmr 1.17 (d, J=6Hz, 3H, C(1)CH_3), 1.61 and 1.68 (s, 9H, CH_3C=), 1.85 (s, 1H, exchanges with D_2O, OH), 1.80-2.33 (m, 8H, CH_2), 3.78 (m, 1H, C(1)H), and 5.14 (bs, 2H, vinyl H). Tlc analysis showed a single spot (Rf 0.27; 10:1, benzene-ethyl acetate). Glc analysis showed a single peak (tr 10.99 min; System A, 125°).

1,5,9-Trimethyl-4(E),8-dodecadien-1-yl p-Toluenesulfonate (66). Alcohol 65 (40 g, 0.204 mmol) in 640 ml of dry pyridine was treated with p-toluenesulfonyl chloride (79.4 g, 0.416 mmol) at 0°. The mixture was left to stand in the refrigerator at 4° for 48 hr and poured into 650 g of ice-cold water. The product was extracted with benzene several times, the combined extracts then washed consecutively with cold 50% hydrochloric acid, 10% sodium bicarbonate, and finally water. Drying and concentration in vacuo afforded the desired sulfonate 66 as a pale yellow liquid (69.5 g, 97%): ir 5.99 (w), 6.21 (m), 6.83 (s), 7.27 (vs), 8.43 (vs), 8.53 (vs), 9.12 (s), 10.99 (vs), 11.24 (vs), 12.29 (s), 13.66 (s), and 15.04 (s); nmr 1.31 (d, J=6 Hz, 3H, C(1)CH_3), 1.57, 1.64 and 1.72 (s, 9H, CH_3C=), 1.85-2.22 (m, 8H, CH_2), 2.43 (s, 3H, C(4')CH_3), 4.66 (m, 1H, C(1)H), 5.07 (bs, 2H, vinyl H), 7.38 (d, J=8 Hz, 2H, aromatic protons), 7.87 (d, J=8 Hz, 2H, aromatic protons). Tlc analysis showed a single spot (Rf 0.52; 15% ethyl acetate in hexane).

1,5,9-Trimethyl-4(E),8-dodecadien-1-nitrile (67). Into a solution of tosylate 66 (69 g, 0.197 mol) in 650 ml of HMPA was added sodium cyanide (12 g, 0.2955 mol) and the mixture stirred under nitrogen for 2 hr at 39-42°. The mixture was allowed to reach room temperature, treated with 650 g of ice-cold water, and extracted with hexane (3 x 200 ml). The combined extracts were washed with water (2 x 300 ml), dried, and concentrated in vacuo to give 39.5 g of a

clear liquid consisting of about 80% of the nitrile and other unidentified impurities as judged by tlc analysis (Rf 0.57; 15% ethyl acetate in hexane) and glc analysis (tr 10.5 min; System B, 165°). The ir spectrum showed bands at 4.46 (w), 4.66 (w), 5.75 (w), and 5.99 (w).[98] No further purification was carried out.

2,6,10-Trimethyl-5(E),9-undecadien-1-oic acid (68). The crude nitrile 67 (39 g) prepared above, ethylene glycol (500 ml), and sodium hydroxide (51.7 g) were mixed and heated with stirring for 2 hr at the reflux point (175°). The mixture was allowed to reach room temperature, diluted with water (500 ml), and extracted with CH_2Cl_2 to remove non acidic impurities. The aqueous solution was treated with 50% HCl to pH 2 and extracted again with dichloromethane (3 x 300 ml). The combined acidic extracts were washed with water, dried, concentrated *in vacuo* and distilled through a short path column to furnish 32 g (72% overall yield from geranylacetone) of pure acid 68 as a colorless mobile liquid: bp 110°/0.075 mm [lit.[77] 131-132°/0.15 mm]; ir[77] 3.33 (vs, OH), 5.85 (vs, C=O), 7.78 (m), 8.06 (s), 9.05 (m), 10.64 (s), and 11.83 (w); nmr[77] 1.19 (d, J=7 Hz, 3H, $C(2)CH_3$), 1.61 and 1.68 (s, 9H, $CH_3C=$), 1.67 (m, 2H, $C(3)CH_2$), 2.01 (m, 6H, $CH_2C=$), 2.5 (m, C(2)H), and 12.03 (s, 1H, OH). Tlc analysis showed a single spot (Rf 0.26;

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85:15:1, pet-ether-ether-acetic acid). Glc analysis showed one peak (tr 6.3 min; System B, 220°). The CIMS showed a molecular ion peak at m/e 225 (base peak) and significant fragmentation peaks at 207, 181, 169, and 143.

N,N,2,6,10-Pentamethyl-5(E),9-undecadien-1-oic amide (59b). Into a stirred solution of 1,1-carbonyldiimidazole[78b] (34 g, 0.218 mol) in 500 ml of dry CH_2Cl_2 was injected dropwise a solution of 2,6,10-trimethyl-5(E),9-undecadien-1-oic acid 68 (21.4 g 0.0955 mol) in 25 ml of dry CH_2Cl_2 at 0°. The mixture was refluxed until all evolution of CO_2 ceased (about 15 min). The resulting solution was saturated with anhydrous N,N-dimethylamine, stirred for 1 hr at 0°, and concentrated in vacuo. The residue was taken up in 300 ml of hexane, washed with 5% hydrochloric acid (200 ml), 5% sodium hydroxide (200 ml), water (200 ml), and finally dried over sodium sulfate-magnesium sulfate. Concentration in vacuo and distillation through a short Vigreux column under vacuum afforded 22.161 g (92%) of amide 59b as a colorless mobile liquid: bp 100-102° (0.075 mm); ir (No.3) 5.92 (s, C=O), 6.64 (s), 6.85 (s), 7.12 (s), 7.94 (m), 8.72 (m), 9.05 (m), and 9.48 (w); nmr 1.09 (d, J=7 Hz, 3H, $C(2)CH_3$), 1.60 and 1.68 (s, 9H $CH_3C=$), 1.67 (m, 2H, $C(3)CH_2$), 1.78-2.40 (m, 6H, $CH_2C=$), 2.74 (m, 1H, C(2)H), 2.96 and 3.03 (s, 6H, $N(CH_3)_2$), and 5.13 (bs, 2H, vinyl H). Tlc analysis showed one spot (Rf 0.25; 20% ethyl acetate in hexane). Glc analysis showed one peak (tr 12 min; System A, 175°).

The CIMS showed a molecular ion peak at m/e 252 (base peak).

Anal. calcd for $C_{16}H_{29}NO$: C, 76.44%; H, 11.63%; N, 5.57%. Found: C, 76.18%; H, 11.54%; N, 5.62%

N,N,2,6,10-Pentamethyl undecan-1-oic amide (59c). Amide 59b (2.514 g, 10 mmol) in 100 ml of dry ethanol was hydrogenated in the presence of 10% palladium-on-charcoal (125 mg) as described for the preparation of hexahydro ethyl farnesoate 36 to afford, after bulb-to-bulb distillation (97°/0.025 mm), 59c as a colorless mobile liquid (2.49 g, 97.5%). In another preparation, 3 g of 59b gave 2.9 of 59c (95%): ir 6.04 (s, C=O), 6.67 (sh), 6.78 (m), 7.41(w), 7.94 (w), 8.62(w), 8.93 (m), 9.05 (sh), and 9.48 (w); nmr 0.87 (d, J=5.5 Hz, 9H, C(6,10,10)CH_3), 1.05 (d, J=6.5 Hz, 3H, C(2)CH_3), 0.98-2.00 (m, 14H, CH_2 and C(6,10)H), 2.75 (m, 1H, C(2)H), 2.97 and 3.07 (s, 6H, $N(CH_3)_2$). Tlc analysis showed a single spot (Rf 0.35; 20% ethyl acetate in hexane). Glc analysis showed one peak (tr 15.5 min; System A, 160°). The CIMS showed a molecular ion peak at m/e 256 (base peak).

Anal. Calcd for $C_{16}H_{33}NO$: C, 72.23%; H, 13.02%; N, 5.48%. Found: C, 75.30%; H, 13.01%; N, 5.53%.

N,N-Dimethyl-1-choro-2,6,10-trimethylundeca-1-en-1-amine (61c). This compound was prepared by reacting amide 59c (3.886 g, 15.21 mmol) with phosgene (143 g, 1.43 mol) as described for the preparation of N,N-dimethyl-1-choro-2-methyl propenylamine (59a). Phosgene was bubbled in for 1 hr at 0°, 4hr at ambient temperature, and the mixture stirred for 19 hr at 25° to afford 2.029 g of recovered starting amide, and 2.2102 g (99%, based on recovered starting material and nmr analysis) of the desired product 61c as a pale yellow liquid: the nmr spectrum (No 23) showed 6.1% of starting material and peaks at 0.87 (d, J=5.5 Hz, C(6,10,10)CH_3), 1.0-1.7(m, 12H, CH_2, C(6, 10)H), 1.75 (s, 3H, allyl CH_3), 2.18 (m, 2H, CH_2C=), and 2.39 (s, 6H, $N(CH_3)H$).

3-(2,6,10-Trimethylundeca-1(E),5(E),9-trienyl)-2-methyl-2-(4,8-dimethyl-1-nonyl)-cyclobutanone (53c). This compound was prepared from 1.25 g (5.724 mmol) of tetraene 63 (2Z:2E, 6:94), 1.139 g (5.85 mmol) of silver tetrafluoroborate, and 1.9 g (6.94 mmol) of α-chloro-enamine 61c as described for the synthesis of cyclobutanone 53a. The crude mixture, after removal of volatile contaminants under high vacuum, afforded 2.75 g of a light red oil. Analytical tlc (2% ethyl acetate in hexane) of this material revealed the presence of one product (Rf 0.24), amide 59c (Rf 0.00), and starting tetraene 63 (Rf 0.77). Part of this mixture (400mg) was subjected

to preparative tlc (5% ethyl acetate in hexane). Extraction of the middle band (Rf 0.60) provided 150 mg (42%) of the expected compound as a colorless mobile liquid. Glc analysis (System A, 240°) of the purified product revealed the presence of six major components (Fig. 51A) in the ratio and with the retention times indicated below.

Component	Fig. 51A peak	Retention time (min)	Relative percentages
Unidentified		7.95	2.94
3(Z)	a	8.70	54.84
Unidentified		10.01	3.20
3(E)	b	10.65	30.94
Unidentified		11.27	3.87
Unidentified		12.90	4.21

The stereochemical assignment is discussed in chapter 2, section C. The remaining part of the crude reaction mixture (2.35 g) was chromatographed on silica gel (40 g, 26.5 x 2 cm column). The order of eluent solvents was pet-ether (500 ml), 1% ethyl acetate in pet-ether (400 ml), and 2% ethyl acetate in pet-ether (300 ml). The last 100 ml of solvent eluted the expected compound in the ratios and percentages indicated in the following table.

Fraction	Weight (mg)	Ratio 3Z:3E	% in total mixture
1	7	30:70	67
2	22	33:67	73
3	49	32:68	77
4	72	40:60	85
5	111	47:53	86
6	127	56:44	95
7	181	58:42	94
8	160	60:40	81
9	95	59:41	83
10	84	66:34	84
11	100	74:26	70
12	17	58:42	30

Fraction 3 had essentially the same ir spectrum (No 6) as the mixture isolated by tlc. This ir spectrum showed a strong four member ring carbonyl band at 5.6μ. Although the nmr spectrum of these two mixtures were similar, the ratio of the cyclobutyl methyl singlets at 1.03 and 1.21 ppm were different. In the mixture prepared by tlc (nmr No 24) the signal at 1.21 ppm was larger than that at 1.03. These signals were approximately equivalent in fraction 3 (nmr No 25). Absorptions common in the nmr spectra of the two fractions were as follows: 0.87 (d, J=5.75 Hz, 9H, CH_3C), 0.96-1.54 (m, 14H, CH_2C, CH), 1.62

and 1.68 (s, 12H, CH_3C=), 1.70-2.40 (m, 8H, CH_2=), 2.60-3.34 (m, 3H, cyclobutyl C(3,4) protons), and 5.17 (m, 3H, HC=). Preparative glc (System E, 200°) afforded mixtures consisting mainly of one isomer (a,b in Fig. 51A,B) contaminated with 20% of the other. The infrared spectrum of these purified isomers showed a strong band at 5.6 μ. High resolution mass spectrometry[99] of each enriched isomer gave a molecular weight of 428.4016 (calculated for $C_{30}H_{52}O$, 428.4018). The fragmentation pattern was very similar for both fractions with only slight variation in intensities of the peaks being observed. Significant peaks were observed at m/e 410, 386, 359, 343, 277, 249, 245, 137, 123, 109 and 107. The base peak varied in these spectra. The fraction with the ratio of isomers a/b, the base peak and the number of spectra having this base peak are given as follows: 80/20; 123,1; 109, 2; and 107, 4. 20/80; 137, 2; 109, 2; and 107, 1.

Attempted preparation of 3-(2,6,10-Trimethylundeca-1(E),5(E),9-trienyl)-2-methyl-2-(4,8-dimethyl-3(E),7-nonadienyl)-cyclobutanone (53b). The two steps described for the preparation of dimethylcyclobutanone 53a were employed for the preparation of 53b. Phosgene (180g, 1.8 mol) was bubbled through a solution of amide 59b (2.5 g, 9.9 mmol) for 5 hr at 0°. The mixture was stirred 5 hr at 0°

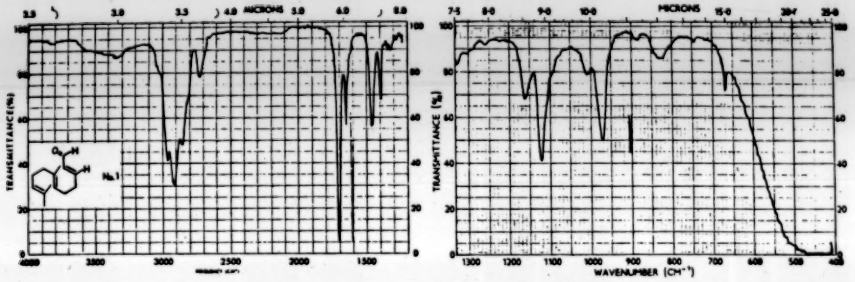
(99) These spectra were kindly provided by Prof. A. Burlingame, Space Sciences Lab., Berkeley, using an AEI-MS902 spectrometer.

and 10 hr at 20°. The product of this step (formation of α-chloroenamine) was treated with a solution of tetraene 63 (1.825 g, 8.4 mmol) and silver tetrafluoroborate (2.5 g, 12.8 mmol) at -78° to furnish, after 1 hr at room temperature, 3.15 g crude reddish oil. Analytical tlc (5% ethyl acetate in hexane) of this mixture showed a major spot (Rf 0.81, starting diene), polar compounds at the origin and two minor products (Rf 0.18, 0.28). This material was chromatographed on silica gel (30 g, 2 x 24 cm column), eluting with hexane (100 ml) and 1% ethyl acetate in hexane (400 ml). The first 100 ml of the polar solvent eluted 1.612 g of starting tetraene 63, while the last 200 ml afforded 275 mg of a light yellow liquid. The infrared spectrum showed a strong band at 5.59, characteristic of cyclobutanones, plus other strong bands at 5.83 and 6.08. Glc analysis (System A, programmed 150-250°, 4°/min), consisted of multiple peaks in the region where C_{15} compounds generally appear (tr 3.38-12.38 min), and four peaks in the region where the C_{30} compounds appear (tr 27.26, 16.47%; 28.61, 46.45%; 29.33, 14.14%; 30.98, 22.93%). This product was bulb-to-bulb distilled to give 230 mg of a light yellow liquid (ir: bands at 5.59, 5.83, and 6.08; glc system above tr 3.38-12.38 min), and a yellow residue which was chromatographed on neutral alumina (2 g, 0.8 x 2 cm). The product was eluted with 10 ml of hexane (Fraction A, 9 mg) and 10 ml of 3% ethyl acetate in hexane (Fraction B, 12 mg). Glc analysis (System A, 240°) showed that

both fractions consisted of four major components with different ratios (Fig. 50B,C). The retention times in this system and percentages are given below.

Retention time (min)	% in 9 mg Fraction (A)	% in 12 mg Fraction (B)
10.95	23.38	9.21
13.18	48.68	44.64
14.44	13.04	12.09
17.44	14.40	34.06

Both fractions had identical ir (No.5): 5.88 (s), 6.17 (s), 6.90 (s), 7.94 (w), 8.05 (w), 8.44 (w), 9.09 (w), 10.42 (m), and 12.2 (m). The nmr spectra (No. 20 and 21 for fractions A and B, respectively) of these fractions are reproduced in this thesis. Aliquots of fraction A and B were combined and analyzed by GC-mass spectrometry. The spectra recorded are reproduced in Fig.51.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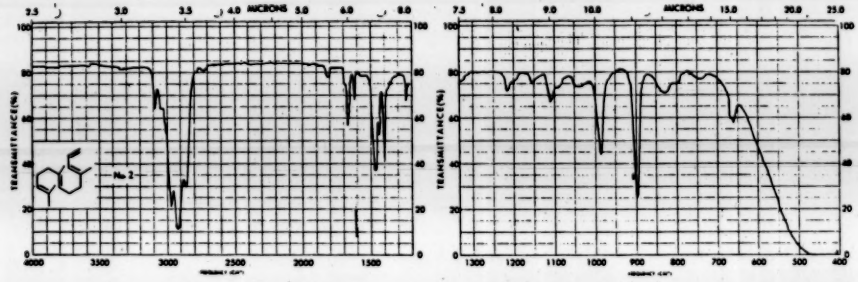
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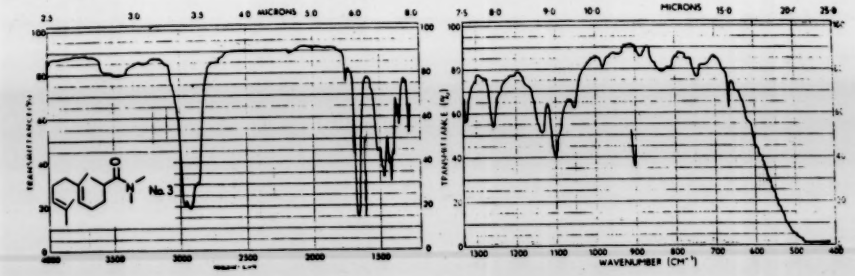
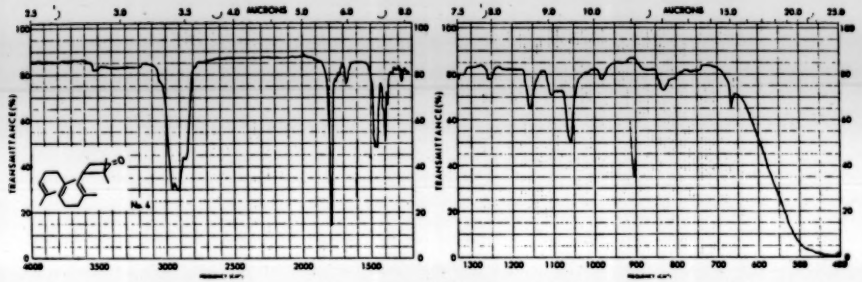
TRANSMITTANCE(%)
No.2


MICRONS
WAVENUMBER (CM⁻¹)
No.3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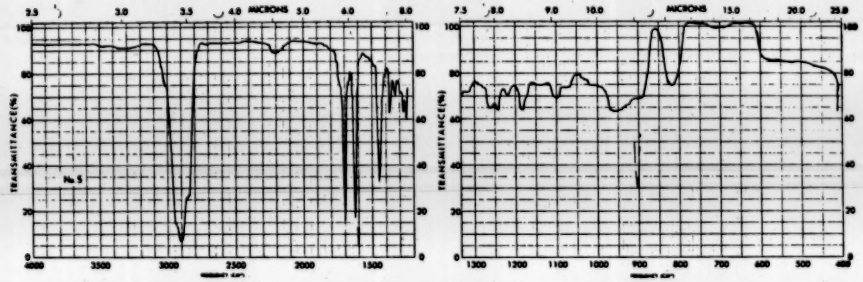
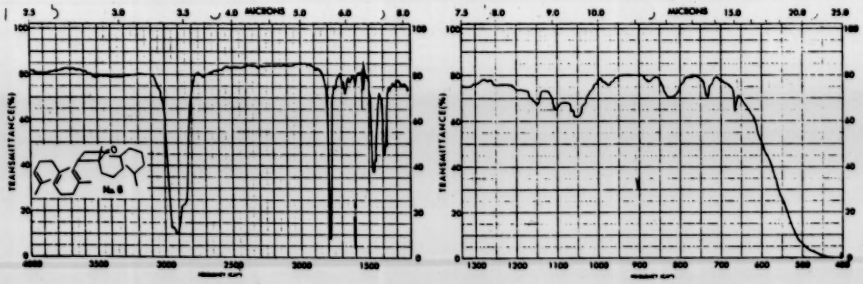
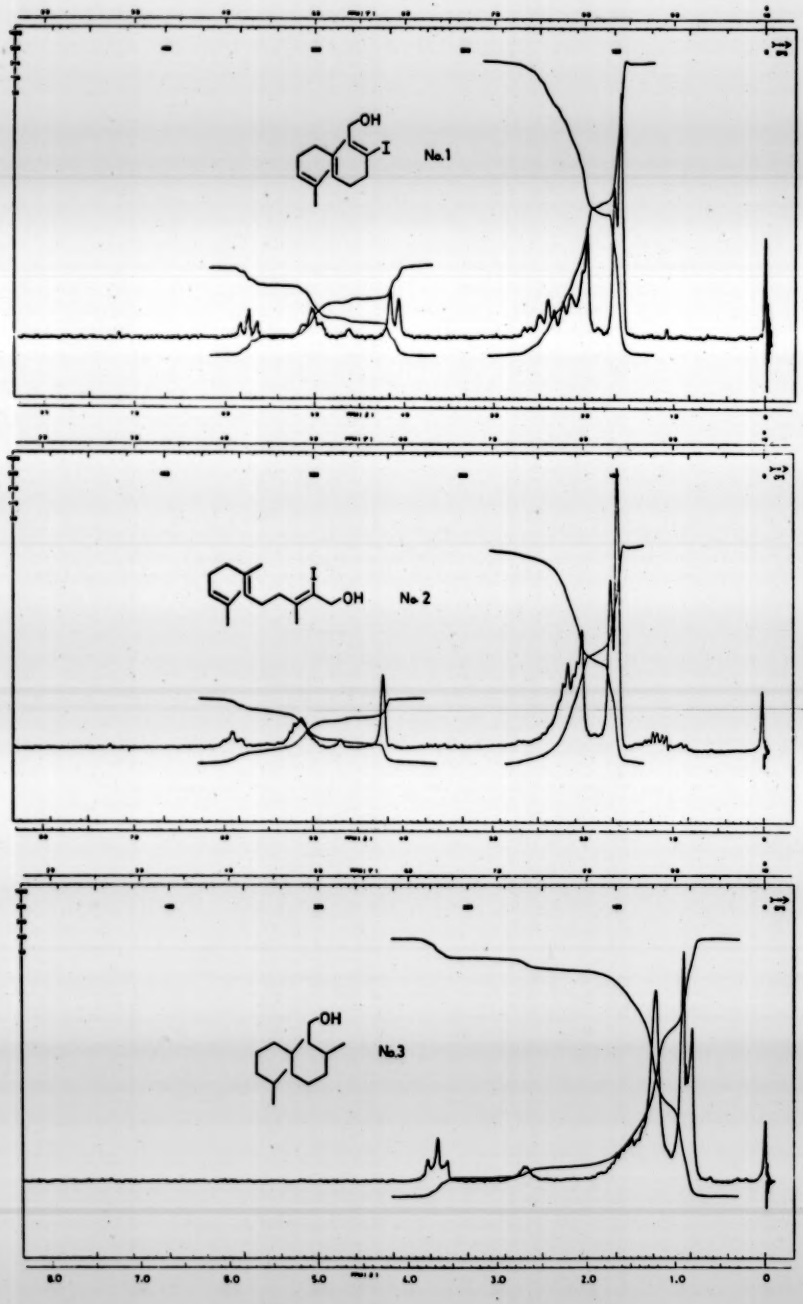
TRANSMITTANCE(%)
No. 4


MICRONS
TRANSMITTANCE(%)
No. 5


MICRONS
TRANSMITTANCE(%)
No. 6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I
No.1
I
OH
No. 2
OH
No.3
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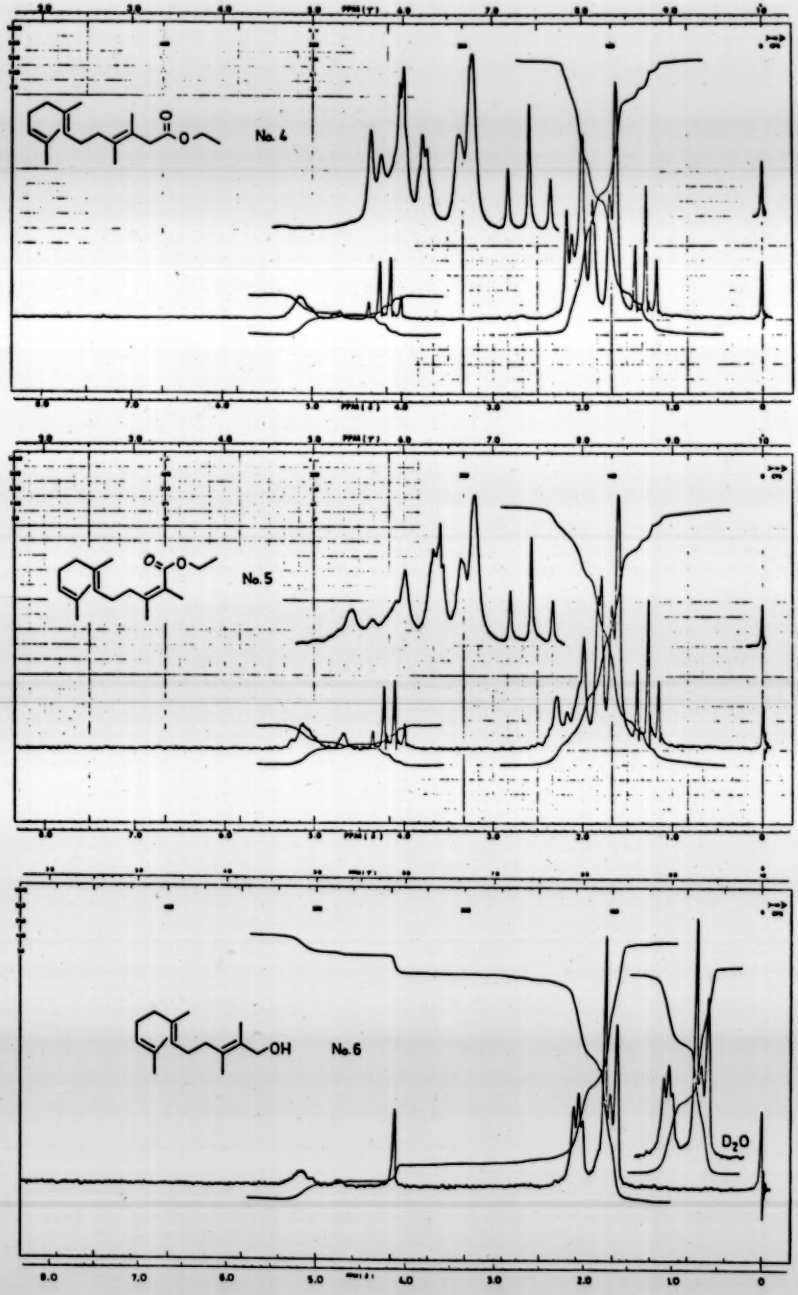
2.0
1.0
0


No. 4
No. 5
No. 6
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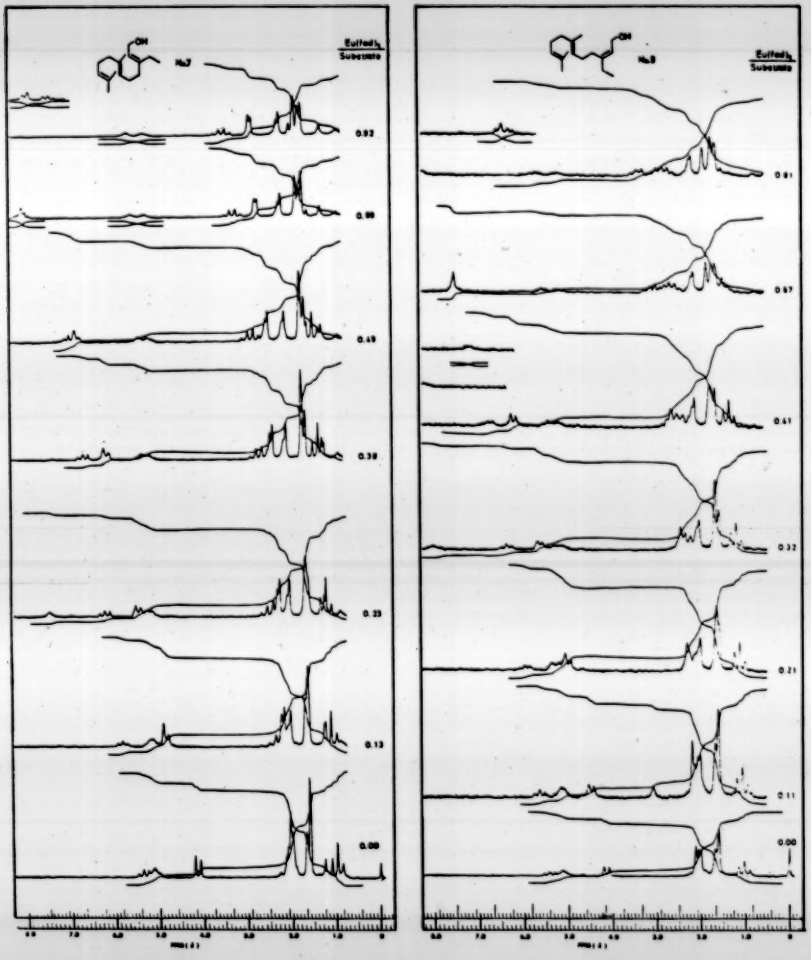
D2O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Substrate
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0.49
0.39
0.33
0.13
0.00
OH
No.8
Eu(fod)3
Substrate
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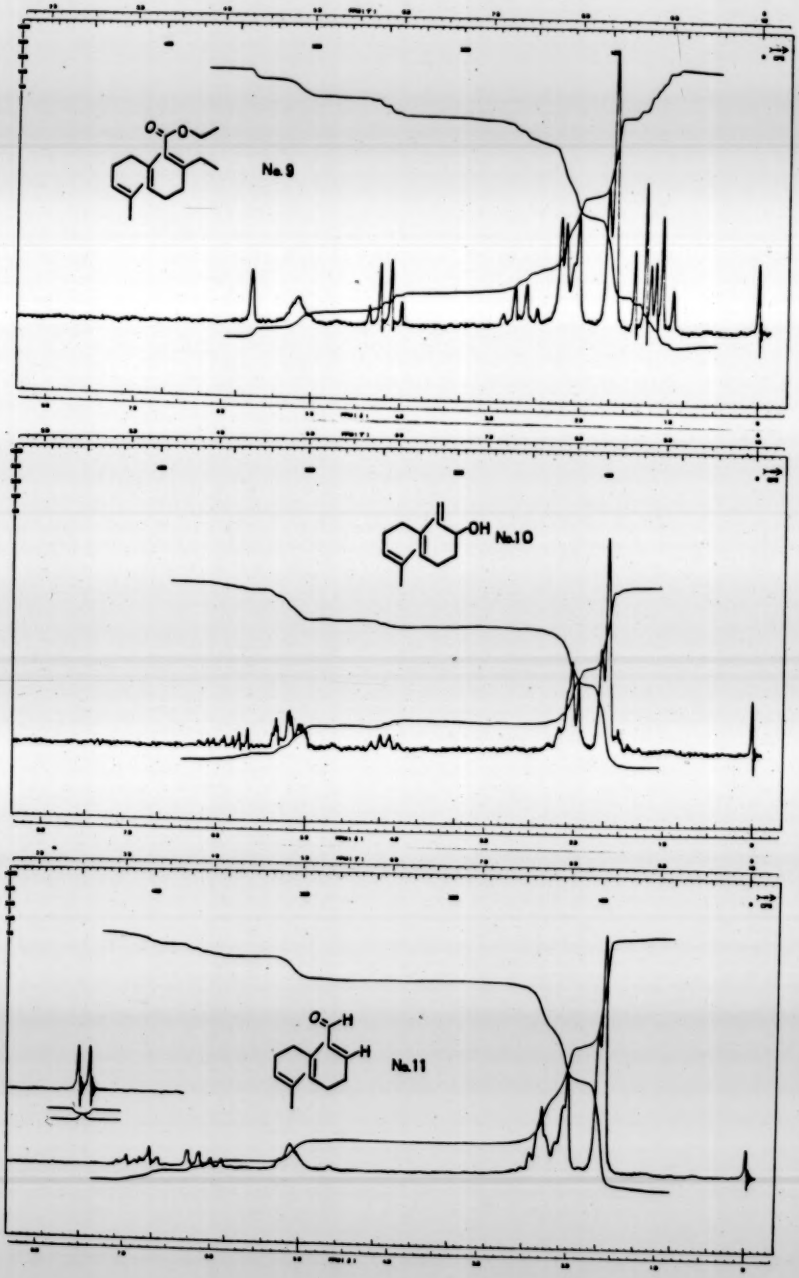
0.67
0.41
0.32
0.21
0.11
0.00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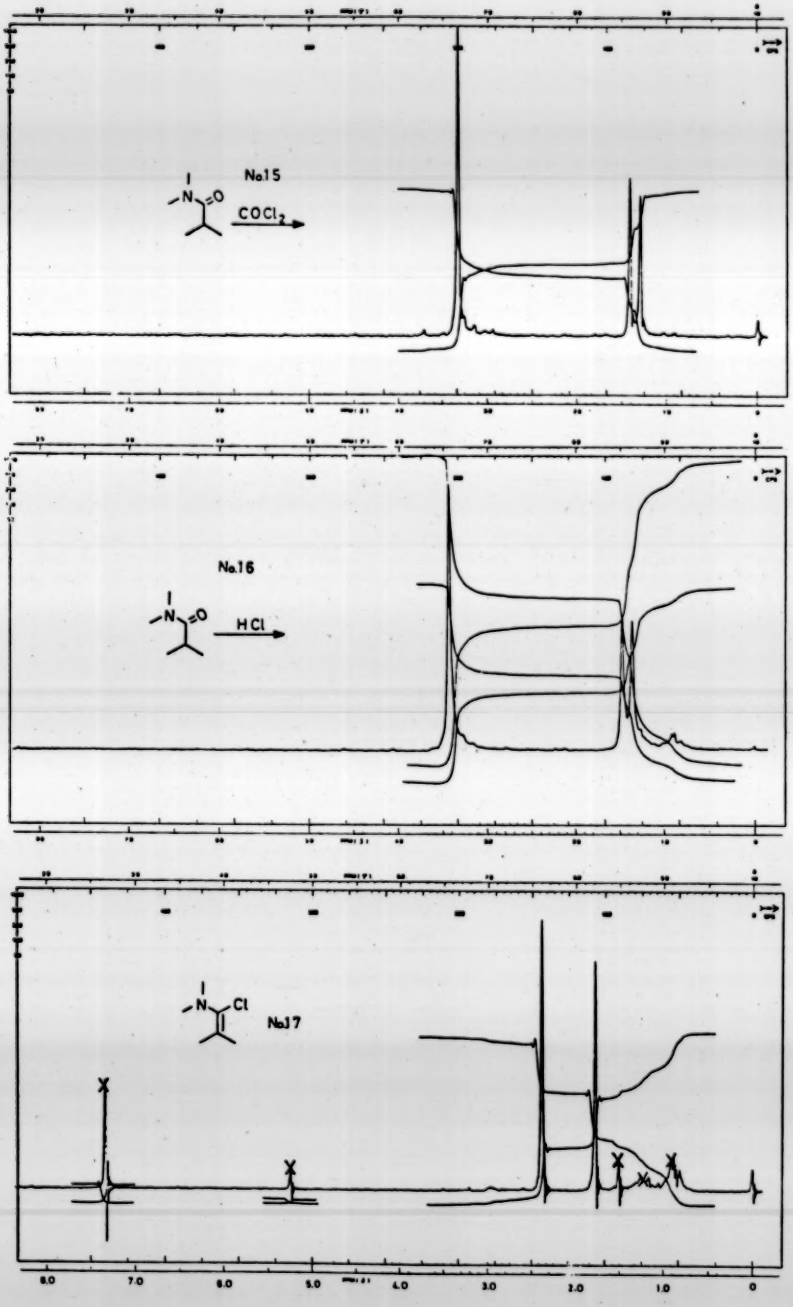
No.10
H
H
No.11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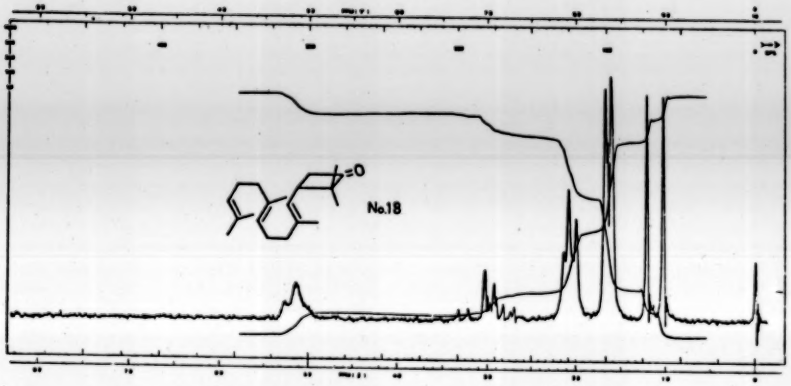
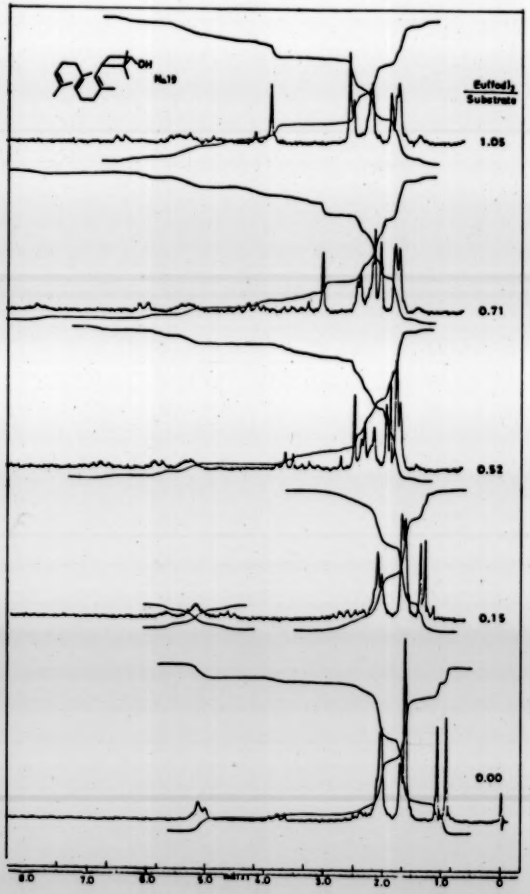
1.0
0


=O
No.18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No.19
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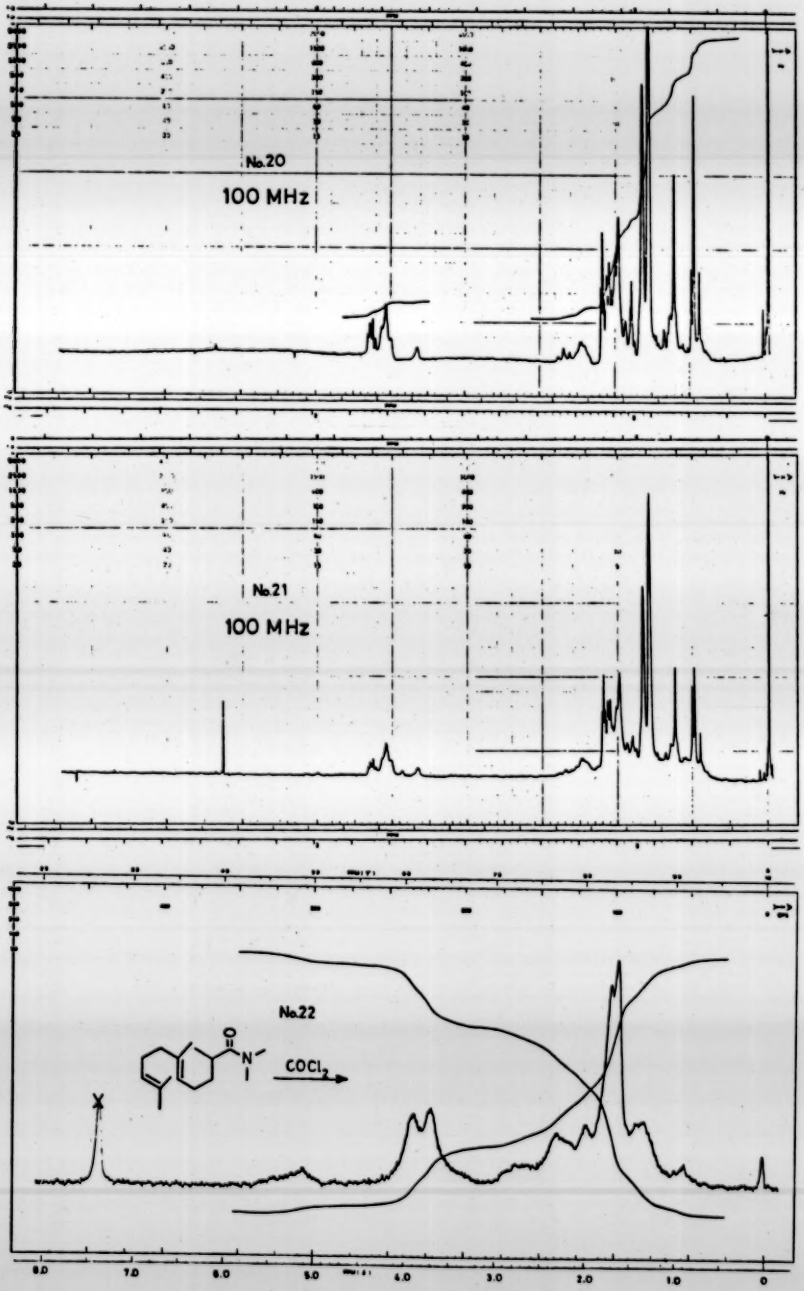
0.52
0.15
0.00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No.21
100 MHz
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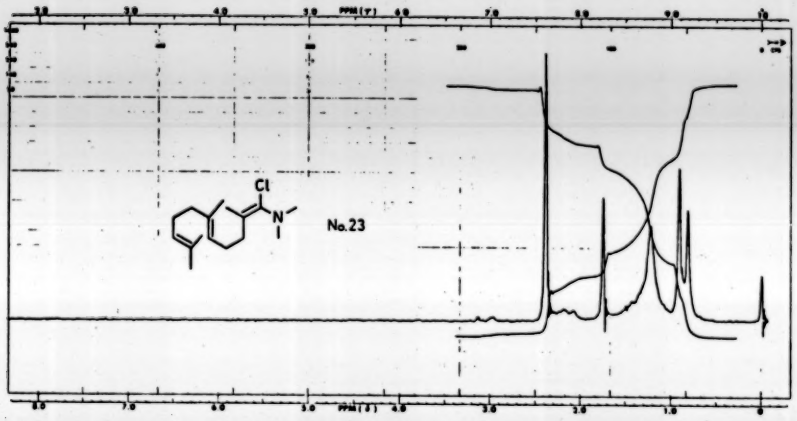
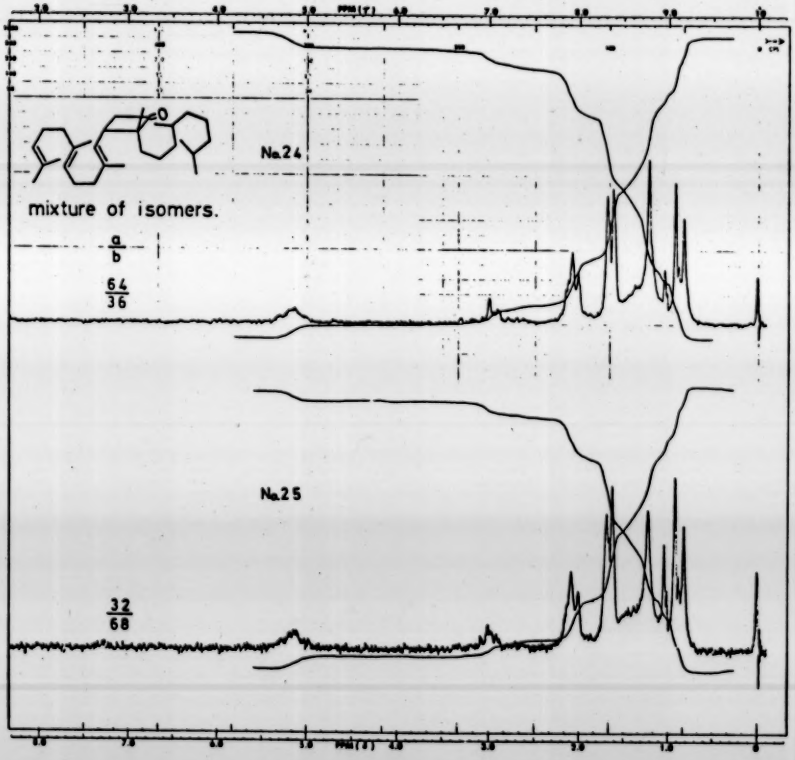
1.0
0


Cl
N
No.23


O
No.24
mixture of isomers.
a
b
64
36
No.25
32
68